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Use of whey protein nanoparticles for the encapsulation and sustained delivery of beta-carotene and zinc micronutrient

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Use of whey protein nanoparticles for the encapsulation and sustained delivery of β-carotene and zinc.

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List of Abbreviations

WPI: Whey protein isolate

WPC: Whey protein concentrate

WPH: Whey protein hydro-lysate

BC: Beta carotene

DCM: Dichloromethane

ALA: Alpha lactalbumin

BLG: beta-lactoglobulin

IgG: immunoglobulin G

BSA: Bovine serum albumin

BCAA: Branched chain amino acid

ALG: alginate

XRD: X-ray diffraction

SDP: Spinning disk processing

EDX: Energy Dipersive Spectroscopy

UV: Ultra violet Spectroscopy

FTIR: Fourier Transform Infrared Spectroscopy

SEM: Scanning Electron Microscope

TEM: Transmission Electron Microscope

HPLC: High performance liquid Chromatography

BOD: Biochemical oxygen demand

DLS: Dynamic Light scattering

ABSTRACT

Nanoparticles prepared from whey protein isolates can be used for encapsulation and sustained delivery of β-carotene and zinc (II) ions. The loaded whey protein nanoparticles were prepared by the pH cycle method at aggregation pH 6 and 22 h ageing time in the presence of calcium ions. Morphology, particle size, stability and release profiles at neutral and acidic conditions of the loaded whey protein nanoparticles were evaluated. The results illustrated that whey protein nanoparticles were able to encapsulate β-carotene molecules in spherical compact structures of around 400 nm that can protect sensitive β-carotene and limit its susceptibility to auto-oxidation reactions when compared to control samples. Moreover, Whey protein nanoparticles showed higher release profiles at neutral conditions compared to acidic conditions even in the presence of proteo-lytic enzymes. These findings confirmed that whey protein nanoparticles can be used to encapsulate, protect and enhance the release profile of sensitive biological active ingredients and micronutrients to be used for food as well as pharmaceutical applications.

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Chapter 1: Introduction

1.1. Whey protein

1.1.1. Overview

Milk Constituents have recently started to be considered functional foods due to their beneficial effects on the human body. Among these constituents, "whey" has been recognized as one of the most nutritious and valuable milk components. By definition, whey is the liquid by-product produced during cheese-making and casein manufacture in the dairy industry. It was discovered about 3000 years ago when the stomachs of calves were used to store and transport milk. During this period, the enzyme Chymosin (rennet) that was naturally present in the cell lining of calves' stomachs, caused milk coagulation, leading to the formation of a solid mass (i.e. curd), while "whey" was the remaining liquid [1]. Historically, whey was considered a waste by cheese-makers and casein manufacturers and they tried different approaches for disposing of it, like discharging into lakes or rivers, spraying onto fields, discharging into the municipal sewage system or selling it as animal feed.[1] All the previous methods were based on the fact that cheese manufacturer believed that whey is a waste product with little value, and that it should be disposed of by the most economical methods. However, disposing of very high amounts of whey by these methods raised different environmental considerations due to the very high BOD value (i.e. 175-fold higher than normal sewage) of whey (Biochemical oxygen demand (BOD) is the amount of dissolved oxygen required by aerobic organisms to break down organic material present in a given water sample at certain temperature over a specific time period. It is important as it is one of the parameters that assess the water quality)[2], therefore different countries started to put tighter restrictions on whey disposal methods.[1]

There are two main types of whey, there is sweet whey and acidic whey, and they differ in the manufacturing process. As sweet whey is produced Sweet whey is manufactured during the making of rennet types of hard cheese like Swiss cheese or cheddar. While Acid whey is a liquid by-product produced during the making of acid types of dairy products like cottage cheese or strained yogurt. [3]

Within this context, manufacturers started to work on different whey-processing techniques to benefit from it and at the same time limit down its environmental hazard. These techniques involve cost-effective and efficient unit processes for concentration, conversion, fractionation and dehydration of whey, along with the invention of new biochemical technologies for the production of new high value whey-based ingredients as nutraceuticals or functional foods for different food industrial applications. [1]

This breakthrough in whey protein utilization was driven by the full understanding of its chemical, physical, biological and nutritional properties.

1.1.2. Composition:

Whey powder contains highly nutritious components like biologically active proteins (i.e. alpha-Iactalbumin (ALA), beta-lactoglobulin (BLG), bovine serum albumin (BSA), immune-globulins (IgG)), also, there are some minors proteins like macro-glycopeptides, lactoferrin and lacto peroxidase along with other significant amount of vitamins, minerals and fats. Among these components, whey proteins are recognized as the most valuable, constituting about 20% of the whole protein content of milk, while casein accounts for the remainder. [4] Table 1-1. Shows the total composition of whey;

Table1-1. Typical composition for Whey powder [5]

Total solids	**96-97%**
Lactose	70-75%
Total protein (N x 6.38)	10-13%
Minerals	
Ash	7-12%
Calcium	4500 mg/kg
Vitamins	
Thiamine	0.4-0.6 mg
Riboflavin	2.3-2.5 mg
Pyridoxine	0.4-0.6 mg

Indeed, whole protein content presents in whey constitutes about 20% of the whole protein content of milk, and they are responsible for the outstanding

attributes of whey. Nowadays, whey is considered a popular dietary protein supplement that is supposed to provide immune modulation, antimicrobial activity, improved muscle strength and, and prevention of osteoporosis and cardiovascular disease.[6][7]

Protein content

All the protein components present in whey powder provide high levels of essential and branched chain amino acids (BCAA); at the same time, they provide some pharmacological activities that are beneficial to human being.

Table 1-2 presents the functions and abundance of different types of protein present in whey:

Table 1-2. Individual whey proteins and their functions and average concentration [7]

Protein	Function	Average concentration
beta-Lactoglobulin	• Binds fat soluble vitamins (K,E,D,A), to enhance their bioavailability • Essential and branched chain amino acids source. • Spares glycogen and muscle during heavy exercises	50-55%
alpha-Lactalbumin	• Rich in the essential amino acid tryptophan that aids in regulating sleep, stress and mood. • Main protein present in human breast milk • Essential and branched chain amino acids source.	20-25%
lmmunoglobulins	• Group of proteins (i.e. IgA. IgO, IgE. IgG, IgM and IgG) that are produced by blood plasma cells to help the immune system in identifying and deactivating foreign organisms like bacteria and viruses.	10-15%

	• Enhances the performance of immunity system.	
Glyco-macropeptide	• Free from the amino acid phenylalanine, so it is suitable for infants with phenylketonuria (It is a genetic metabolic disorder that prevents the body from metabolizing the phenylalanine (Phe) amino acid, leading to its accumulation in then converted to phenylketone) • Prevents dental plaque and cavities` formation	10-15%
Bovine Serum Albumin	• Large protein that contain high amount of essential amino acids • Fat binding properties	5-10%
Lacto-ferrin	• Acts as antioxidant in breast milk, saliva, tears and blood • Antibacterial, antiviral, antifungal • Stimulates the growth of gut flora (beneficial bacteria) • Enhances iron bioavailability	1-2%
Lacto-peroxidase	• Inhibits bacterial growth	0.5%

There are three major types of whey protein; concentrate (WPC), isolate (WPI), and hydro-lysate (WPH). Both forms, isolate and hydro-lysate, are advanced formulas derived from the whey protein concentrate after additional processing steps. These steps result in products that differ from whey concentrate in many important characteristics. Table 1-3, shows the

percentage of protein in each type; where whey protein concentrate has around 29%–89% protein by weight and low level of cholesterol and fat, however, it has higher levels of lactose and other bioactive compounds when compared with the other formulas.

On the other hand, whey protein isolate contains more than 90% protein by weight, as it is subjected to further purifying steps to eliminate the lactose, cholesterol and fat in order to maximize the protein yield.[8]

Table 1-3. Percent composition of different types of whey protein products[8]***;***

Component	Whey Powder	Whey Concentrate	Whey Isolate
Protein	11 – 14.5	28 – 89	90 +
Lactose	63 – 75	10 – 55	0.5
Milk Fat	1 – 1.5	2 – 10	0.5

The third type of whey protein is the whey protein hydro-lysates which are partially hydrolyzed by exposing the protein to acid, heat, or enzymes that break apart the bonds between amino acids. This makes this type of proteins are more absorbable than a concentrate or isolate. [9]

1.1.3. Processing

Commercially, sweet whey is produced during the cheese-making process when fluid milk, that contains caseins and whey proteins, is turned into cheese after the addition of specific enzymes and bacteria. These bacteria metabolize the milk lactose to generate lactic acid. This lactic acid decreases the milk pH value from 6.7 to around 5.3, which causes the coagulation of casein proteins. The whey proteins don't denature (a process in which proteins lose the quaternary, tertiary and secondary structures which is

present in their original form) at this pH level due to their unique structure, but they get trapped within the casein gel that forms. Cheese producers introduce perforation into this gel In order to release most of the whey and accompanying proteins from the casein curd, the whey is then drained from the curd and gets utilized into a variety of ingredients.

During cheese manufacturing approximately 90% by volume of the entered liquid milk to the plant is converted to whey. And the most profitable solution for the ecological problem caused by whey is to reuse it in other pharmaceutical and food applications. Thus, cost-effective processing of the whey is very importance to the economics of cheese manufacture, because the cost of whey disposal may reach £200 per ton (2006).[10]

Any cheese factory that produces more than $300m^3$ per day of whey can process whey commercially. For other dairy manufacturers who do not produce this volume of scale, it is common to pre-concentrate the whey at the cheese factory and transport it for downstream processing in a dedicated facility.[10]

There are three main ways of whey processing, lactose production, animal feed-stocks and the production of whey powders and individual whey proteins [11].

Figure 1-1. shows the industrial processing of whey. Very long treatment of whey at average temperatures, ranging from 55˚C to 63˚C, and at certain stages of industrial vacuum evaporators the temperature may reach 70˚C, can cause whey protein denaturation. However, applying the low-temperature methodology of vacuum concentration of whey at 20˚C and ultrafiltration at 50˚C, preserves the natural structure of whey proteins in order to benefit from the bio-regulatory and physico-chemical effects of these products.[10]

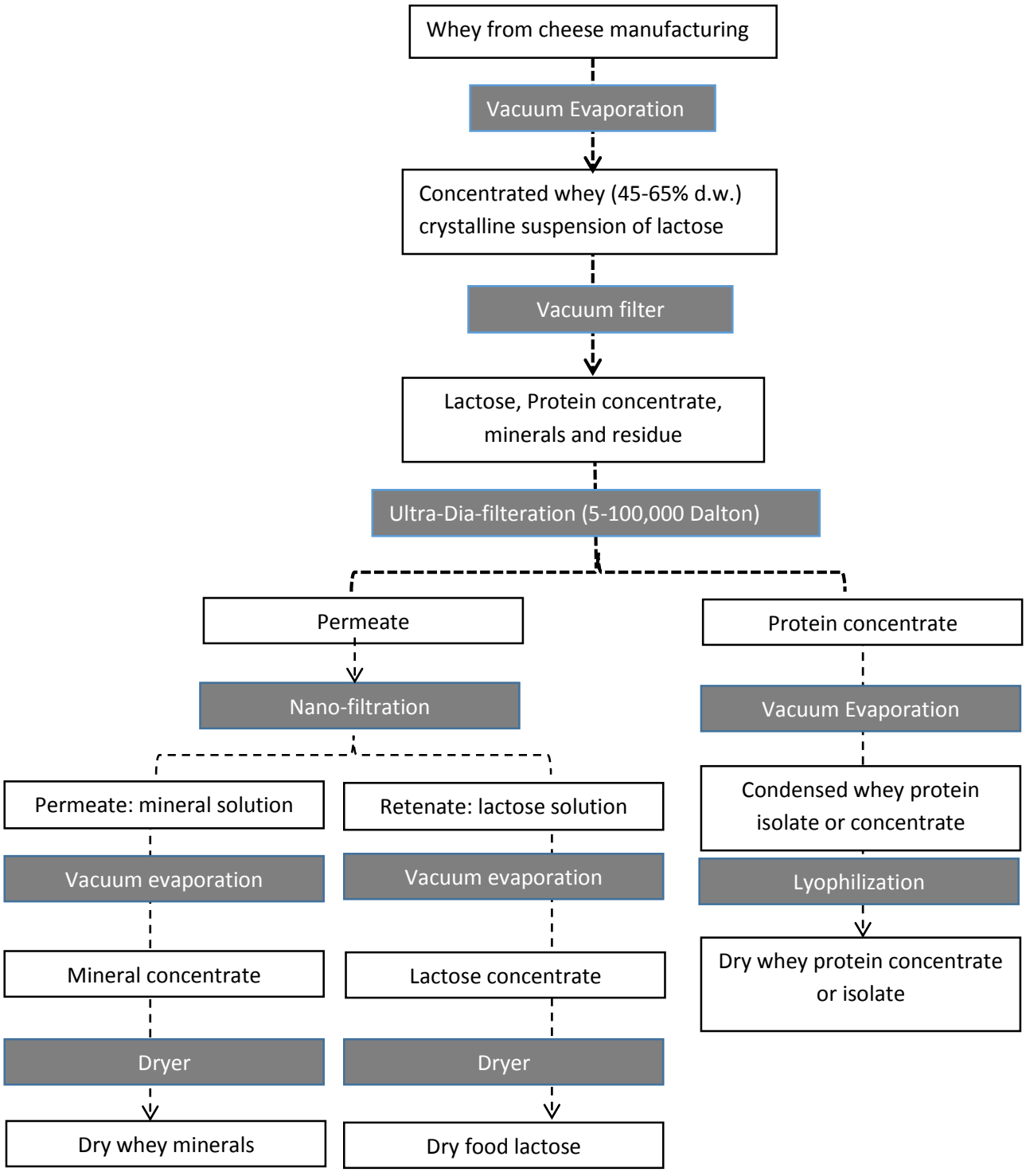


Figure 1-1. Technology of whey processing into pharmaceutical and food products

1.1.4. Pharmacokinetics:

Whey proteins exhibit a highly stable pharmacokinetic profile, as they do not coagulate under acidic environments and they can also resist the destructive action of enzyme Chymosin in the stomach. Lacto-ferrin protein, for example, can survive the stomach acid without being denatured. However, whey proteins can reach the Jejunum quickly so they are considered to be "fast proteins,". After reaching the small intestine, they are hydrolyzed at a slow rate, even slower than that of casein, which allows greater percentage of protein to be absorbed over the whole length of the small intestine. Moreover, whey protein has higher postprandial level for plasma amino acids than casein. [7]

1.1.5. Applications:

Whey contains high levels of protein, vitamins and minerals, and is thus most recognized for its applicability in sports nutrition. Furthermore, whey products could also be used in baked goods, salad dressings, emulsifiers, and medical nutritional formulas. Moreover, Beta-lactoglobulin can be used in infants formulas.

There are other innovative food and non-food applications for whey protein that could be attributed to its superior emulsification and gelling properties. β- Lactoglobulin is the most abundant protein and it has a very effective gelling property. Thus, whey protein gels could be used as pH-sensitive hydrogels (Hydrogel is a three-dimensional structure that has the ability to swell in water and retains a considerable amount of water within its network) for the controlled delivery of biologically-active substances [12].

1.2. Carotenoids

1.2.1. Structure

Carotenoids belong to the unsaturated hydrocarbons class with specific functional end groups and conjugated double bond system. Structurally, carotenoids may or may not have additional oxygen atoms attached. Carotenoids that contain oxygen are named xanthophylls (Figure 1-2) like lutein and zeaxanthin. However, α-carotene, β-carotene, and lycopene are the oxygen free carotenoinds (Figure 2).

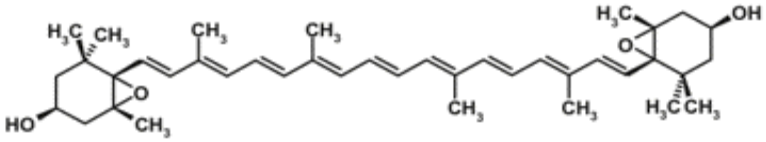


Figure 1-2 Xanthophylls (oxygenated carotenoid)[13]

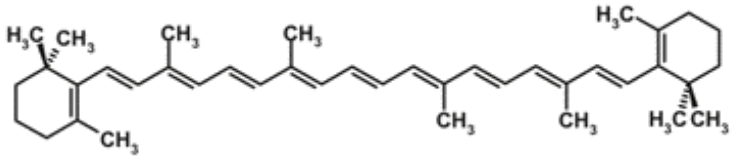


Figure 1-3 Carotene (oxygen free carotenoid)[13]

Carotenoids can also be classified according to their provitamin A activity, where α-carotene, β-carotene, and β-cryptoxanthin, can be converted in the body to retinol and are thus classified as pro-vitamin A carotenoids. On the other hand, Lutein Lycopene, and zeaxanthin carotenoids have no vitamin A activity, thus classified as non- provitamin A.[14]

1.2.2. Color

Structure of carotenoids is responsible for their pale yellow to deep red color. The conjugated double bond system allows free movement of electrons across the hydrocarbon backbone of the molecule. As the number of conjugated double bonds increases, electron movement through the molecule requires less energy to occur. Which allows more absorbance of light from the low frequency end of the visible spectrum, thus compounds acquire an increasingly red color. [14]

1.2.3. Solubility

Carotenoids belong to the fat soluble vitamins category which include also vitamins A, D, E and K these groups of compounds are characterized by limited water solubility, and hence bioavailability. This attribute is due to the presence of long unsaturated aliphatic chains as in some fatty acids. Thus, the biological absorption of carotenoids and the other fat soluble vitamins depend completely on the presence of fats and bile salts. [14]

Functional compounds like carotenoids are usually used as active ingredients in different food stuff. Yet, the hydrophobic property of these ingredients has made their use in different food formulations very difficult. Thus, food technologists started to explore different opportunities through nanotechnology to enhance the hydrophilic character of such functional ingredients and to improve bioavailability. [15]

1.2.4. β- carotene

β-carotene has been identified as the most common carotenoid in vegetables and fruits, and due to its outstanding bright coloring effect, it is introduced to many food, cosmetic and personal care products, in where also the proposed health protective benefit of β-carotene is taken into account. β-carotene is found naturally and at very high amounts in apricot, carrot, dark green leafy vegetables, squash and colored peppers.[16]

1.2.5. Biosynthesis

The biosynthesis of carotenoids in plants is done through the following enzymatic pathway:

Phytoene -->phytofluene --> ζ-carotene --> neurosporene --> lycopene --> γ-carotene and β-carotene

In each enzymatic step starting from phytoene till lycopene, only one double bond is added to the molecule, resulting in lycopene, which is a 13 double bonds symmetrical molecule. After Lycopene synthesis, cyclization enzyme starts to cyclize the end groups, which results in molecules having one beta ring (γ –carotene) another one having two beta rings (β-carotene). Adding oxygen to the molecule results in the formation of xanthophylls [16]

1.2.6. Pharmacological effects

Moreover, Beta-carotene exhibits pro-vitamin A activity and it is also considered a potent biological antioxidant, [16] as it has a very high ability to quench singlet molecular oxygen and free radicals. This effect is achieved by preventing radical chain reaction of lipid peroxidation that takes place in bio-membranes, and scavenging lipid peroxyl radicals to produce stable non-radical products.[14]

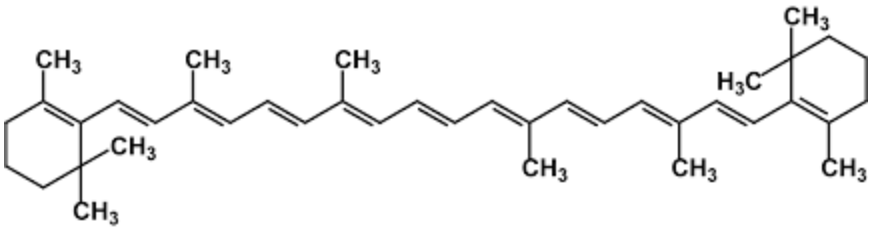


Figure 1-4. Beta-carotene with two cyclohexene type end groups.[17]

The powerful antioxidant property of beta-carotene has led to its ability to reduce the risk of different diseases that are related to cells damage. Many Studies have proven that high consumption of vegetables and fruits containing β- carotene will reduce the risk of developing diseases such as cancer, cardiovascular diseases, photosensitivity and aged-linked disorders, however, recent studies, had referred to a negative relationship between β-carotene consumption and lung cancer and cardiovascular disease in smokers.[18]

1.3. Micronutrients (Trace minerals)

Trace minerals or micronutrients are group of ions (like iron, zinc, manganese, copper and selenium) that the body needs in a very small amount. The averages of recommended dietary allowances (RDA) for trace minerals are between 0.2 - 15 milligrams per day.

1.3.1. Zinc

Zinc is a trace mineral that is needed for the growth and development of nervous, immune and reproductive systems. Indeed, it is essential for many vital processes in the body like protein folding, nucleic acid synthesis, gene expression regulation, and catalysis of more than 100 enzymes. Thus, it is involved in cell division activity and ideal hormone level maintenance.

Zinc also is classified as a potent antioxidant that can scavenge free radicals and active oxygen species that affects cell integrity and induces tissue inflammation. However, many studies had reported that around 40% of the whole population are estimated to be deficient in zinc. And this deficiency is

due to the inadequate consumption of zinc rich food and the low bioavailability. The RDA of zinc is around 2 to 3 milligrams per day for adults. Insufficient intake of zinc is linked to incidence diarrhea, malaria in children, impaired cognitive function, low immunity, infertility, slow wound healing, giving birth to underweight infants and low respiratory tract infections.

Zinc metal ions have a role in β-carotene (pro-vitamin A) metabolism, including its absorption, transport, and utilization [19]. It is therefore recommended to combine zinc ions with β-carotene supplementation.

In conclusion, increasing the dietary intake of beta-carotene and zinc through food formulation enriched with unique nanoparticles systems is of great benefit in improving the micronutrient state in malnourished adults and children specifically in developing countries.

1.4. Statement of purpose:

The scope of this work is to investigate the potential of whey protein isolate nanoparticles for storing carotenoids (i.e. β-carotene) and trace minerals (i.e. zinc), as well as their release, this entails:

- Preparation of β-carotene and zinc loaded whey protein nanoparticles.
- Characterizing the complex by evaluating the morphology, particle size, stability and loading efficiency of the whey protein nanoparticles.
- Studying the release behavior of the beta carotene and zinc from the encapsulated whey protein nanoparticles.

Chapter 2. Literature review:

2.1. Methods for whey protein isolate nanoparticles preparation:

Protein nanoparticles are generally used for encapsulation and delivery of pharmacologically active compounds as they can deliver the active compounds with high ratio of absorption to a particular tissue of the body. Thus, and due to their unique physicochemical characteristics, whey protein nanoparticles could be a good candidate for novel food as well as nonfood applications. Different preparation method for whey protein nanoparticles were proposed by different research groups.

Preparation of whey protein isolate nanoparticles through pH cycle was first carried out by Hélèn *et al* where they formulated controlled size nanoparticles by cross-linking of denatured whey protein strands at low temperature and controlled pH cycle. Heating whey protein at pH 7 and low ionic strength produced soluble polymers, which were acidified then neutralized again to produce nanoparticles. The particle sizes of the produced nanoparticles ranged from 100 to 300 d.nm depending on the aggregation pH (either 5, 5.5 or 6.0), the ageing time at the aggregation pH (0–75 h) and calcium concentration (0, 2.5, 5 mM). It was confirmed that increasing the calcium concentration increased the turbidity and particle size but it decreased the voluminosity of the nanoparticle dispersion. Introducing calcium to the formulation increased the particle compactness with an impact on the delivery and protection abilities of the nanoparticles. On the other hand, the diameter size increased by increasing the ageing time of the formulation, due to the formation of disulfide bonds which were responsible for particle intra -cross links. Finally, the authors suggested using the formed nanoparticles for the encapsulation of aroma and pharmacologically active compounds to benefit from their outstanding abilities. [20]

In 2012, another method of whey protein isolate nanoparticles preparation was proposed by Gülseren *et al.* This entailed diluting whey protein solution by ethanol at alkaline pH, then immediately diluting in buffers (either at pH

7 or 3). The size of the formed nanoparticles ranged from 10-100nm. However the particle size could be modified by a combination of homogenization and heating. There was no substantial difference in the interfacial properties between the nanoparticles prepared by the desolvation method and the corresponding original protein solution which may suggest the use of whey protein nanoparticles to enhance emulsions` stability. [6]

A heat-stable whey protein nanoparticles with very low turbidity level could be formed by following the same method proposed by Zhang et al. The method involved incorporating thermally pretreated whey protein molecules in nano-micelles of water in an oil emulsion to produce whey protein nanoparticles with less than 100nm particle size. The pH of the formula during the preparation had a significant effect on the stability of the final formulation, as nanoparticles produced from neutral protein solution showed higher thermal stability than those produced from acidic solution. Indeed, the main objective of Zhang`s study was to retard the heat induced gelation property of the very tiny whey protein particles (i.e. < 100 nm) to allow its use in clear beverages. [21]

Hebrard et al. used whey protein (WP) isolate and alginate (ALG) for the encapsulation of a valuable type of Saccharomyces boulardii yeasts . The formulated microparticles were able to protect the yeast and control its delivery throughout the gastrointestinal tract. The method used for particles preparation was the extrusion/ cold gelation method by means of an "encapsulator" device which uses a vibrating nozzle to induce laminar jet break up. The results showed that a formula composed of 62 % whey protein to 38 % alginate was able to protect up to 40% of the yeast cells compared with only 10% survival rate for free yeast cells in simulated gastric intestinal fluid, even in the presence of digestive enzymes like pepsin and pancreatin. Coating the WP/ALG microparticles with alginate led to higher survival rate of yeast cells, which could be attributed to the shrinkage behavior of ALG`s matrix in gastro intestinal fluids. This would limit the passage of digestive enzymes through its network leading to a higher survival rate of probiotics. The release of yeast cells from the microparticles was shown to occur due to alginate swelling and whey protein hydrolysis in intestinal fluid. Probiotics

loaded microparticles can be used for many applications in food as well as pharmaceutical industry for better protection of the laded organisms.[22]

2.2. Use of whey protein for encapsulation and drug delivery application:

In 2007, Gunasekaran et al. studied the use of whey proteins as hydrogels and/or nanoparticles systems for the encapsulation and controlled release of pharmacologically active compounds. Indeed, individual whey proteins show high swelling sensitivity towards pHs that are higher than their isoelectric point. Caffeine was used as model drug to examine the controlled release ability of whey protein hydrogels. However the results confirmed that the release of caffeine from hydrogels was slower at pHs lower than the isoelectric pH (the pH at which the net charge of protein equals zero). Sodium alginate coatings can also be used to further control the sustained release behavior of whey protein hydrogels. Gunasekaran et al also formulated beta-lactoglobulin (BLG) nanoparticles by the desolvation method and the average size of the resulted nanoparticles was less than 100nm. Preheating the BLG solution to 60°C before the nanoparticle formation would decrease the average size and improve the uniformity of the yielded nanoparticles. The stability of the nanoparticles suspension at different pHs was investigated and the results revealed that their stability at neutral pH was higher than acidic pH even in the presence of proteolytic enzymes. This could be explained by the highly dense structure of the nanoparticles. [12]

In 2011, Giroux *et al* succeeded in encapsulating a hydrophobic aroma (i.e. ethyl hexanoate) in whey protein nanoparticles through pH cycling method. The size of the produced nanoparticles was less than 300 nm in diameter. Moreover, nano-particulation of lipophilic aroma resulted in better retention of aroma and less mass transfer at the surface of the matrix. The efficiency of encapsulation was tested by comparing the release behavior of aroma from nanoparticles and from native whey protein, however the results showed that the release of aroma from nanoparticles was significantly lower than that from native whey protein which confirms the encapsulation efficiency of nanoparticles. The study had confirmed also that for better

entrapment of aroma in the cross linking network it should be first, added before pH cycling, second, produced at pH 5.0 and 5.5, and third, without calcium addition, to make sure that the structure of the produced nanoparticles is more porous and less compact and hence better entrapment capacity. In conclusion, nanoparticulation of hydrophobic aromas or flavors in whey protein could be used as a method to control their release in low or non-fat foodstuffs.[23]

In the same respect, in 2014, Abbasi et al studied the loading of Vitamin D^3 in whey protein isolate (WPI) nanoparticles prepared by pH cycling at different calcium concentration. The stability of the formula was tested in the presence of oxygen for 7 days. Results suggested that the whey protein nanopartricles had the ability to retain more vitamin D_3 than control samples of native whey protein isolate and of denatured whey protein. The authors confirmed that adding the active compounds before pH cycling led to better entrapment. Adding calcium during nanoparticle preparation resulted in a more dense structure which provided further protection for the entrapped vitamin D_3. The kinetics of vitamin D_3 release during storage time followed a second order model. Results showed that these nanoparticles may be used as enriching agent in different beverages and foodstuffs.[24]

In 2012, Gülseren *et al*, succeeded in incorporating the daily required amount of zinc for adults in whey protein isolate (WPI) nanoparticles by applying the ethanol desolvation method. The volume of ethanol added was 0–3 times the volume of protein dispersion and the desolvation took place at pH 9. However, directly after desolvation, the desolvated solutions were dispersed in acidic water (pH 3). The size of produced nanoparticles was controlled by the amount of ZnCl used and the ethanol to water ratio. It was found to increase by increasing these factors. The encapsulation capacity of whey protein nanoparticles for zinc was studied and the results showed that the amount of zinc retained was high and remained stable for 30 days at 22˚C. In this respect, whey protein nanoparticles could act as a reservoir for sensitive hydrophilic materials, which would allow their addition to different beverages and food products. [25]

Another research group embedded ZnO nanoparticles in a whey protein isolate matrix to provide protection for Zn mineral in gastric pH and improve its bioavailability. Transmission electron microscopy and X-Ray diffraction results confirmed the successful synthesis of ZnO-WPI nano-scaled composite structure of 300 nm average size and uniform mono-dispersal embedment of ZnO in the structure. [26]

2.3. Beta carotene in nano-forms

β-carotene, which is a lipophilic carotenoid is of great interest mainly because it is one of the most effective precursors for vitamin A, and also because it has other potential health effects, such as being a cancer prevention agent, life extender, ulcer and heart attack inhibitor. However, its utilization is limited due to its poor water-solubility and chemical instability. Thus, β-carotene has to be dissolved in oils or other appropriate solvents before being utilized in foods. Moreover, β-Carotene is extremely susceptible to chemical degradation during food processing and storage. Hence, improving the thermal and chemical stability as well as solubility profile is an important challenge for β-carotene utilization in different food and pharmaceutical applications. [27]

Guanghua et al. used human H chain ferritin (rHuHF) which is naturally present with a shell-like structure and reversible dissociation and reassembly properties at different pHs to encapsulate β-carotene and hence enhance its thermal stability and water solubility. High-performance liquid chromatography (HPLC), transmission electron microscope (TEM) and UV/Vis spectroscopy showed that β-carotene molecules were successfully encapsulated within protein cages and each cage contains 12.4 molecules of β-carotene. Moreover the water solubility of β-carotene encapsulated within protein cages was higher when compared with freely oil soluble β-carotene molecules. Additionally, its thermal stability was noticeably improved. This would allow the utilization of β-carotene in food industry. [27]

In the same respect, Zhong et al. tried to encapsulate β-carotene in different food proteins like whey protein isolate, sodium caseinate and soybean protein isolate. The technique used for their preparation was the homogenization-evaporation technique in which β-carotene solution was added to the protein solutions and homogenized at different speeds before the evaporation step taking place to remove the extra solvent. The results of the chemical antioxidant assays showed that the free radical scavenging activity and the reducing power of encapsulated β-carotene was higher than the free form when tested on Caco-2 cells (i.e. human intestinal cell line). Fourier transfer infrared (FT-IR) as well as X-ray diffraction (XRD) findings showed that β-carotene became amorphous after encapsulation in food proteins. The *in-vitro* release studies indicated that β-carotene that was encapsulated in whey protein isolate had the best delivery profile and it was able to survive pepsin in the gastric medium and release the β-carotene in the intestine under the effect of trypsin enzyme. [28]

Yassin et al. encapsulated β-carotene in polysorbate-80-coated poly(d,l-lactide-co-glycolide) nanoparticles to treat epileptic convulsion. The prepared nanoparticles had the ability to target the brain and exhibit high antioxidant activity to treat epileptic convulsions. Solvent evaporation was used to prepare the nanoparticles. The dose in the formula that was shown to be effective in treating epileptic convulsions is 2 mg/kg. However the same "unformulated" amount of β-carotene dose exerted no effect in vivo. The findings proved that β-carotene encapsulated in Tween-80 polymer can be used as a novel anti-convulsant drug with very high stability, bioavailability as well as drug selectivity even at very low doses.[29]

In 2014, Sheng et al. tried to prepare β-carotene molecules as nanoparticles by anti-solvent precipitation with the assistance of ultra-sounds. The workers also studied the effect of different process parameters on the formulation. For example, at an efficient concentration of 5–15 mg/ml, the nanoparticles precipitating under 1 minute ultrasound had smaller size (112–141 nm) than the other nanoparticles that were processed without ultrasonication(144–365 nm). Moreover, without ultrasound, the order of anti-solvent (i.e. water) addition was shown to have a significant impact on the particle size: adding

water to the active solution led to larger particles than adding the active solution to the water. Furthermore, the ratio of active solution to anti-solvent volume affected the particle size considerably, as decreasing the active solution to anti-solvent from 1:1 to 1:4 without ultrasound decreased the particle size from 432 to 223 nm. Finally the produced β-carotene nanoparticles precipitated under ultrasound exhibiting higher dissolution than free β-carotene molecules and nanoparticles precipitated without ultrasound. [30]

Martín et al. used supercritical anti-solvent precipitation technique from an oil-in-water emulsion to prepare a β-carotene nano-suspension with a controlled particle size. The technique was composed of a two-step process strategy. The first one involved a short contact between CO_2 and the emulsion till the saturation of the disperse phase. This was carried out in order to achieve the precipitation by the effect of anti-solvent. On the other hand, the second step involved an extended contact between the emulsion and CO_2 in order to eliminate the organic solvent residual. The produced particle size was less than 400 nm in suspension. Martin et al used a modified edible starch surfactant because it could be used as a coating material for the final dried nanoparticles that were produced by lyophilization technique. However, the results showed that β-carotene got encapsulated in the micelles formed by the surfactant with a final concentration of organic solvent as low as 1 ppm. [31]

Similarly, the preparation of β-carotene nanosuspension using green, solvent free method was studied in 2012 by Tien. The partially water-soluble compound "Triacetin" was used in the nanoemulsion as a dispersed phase. However, the effect of water concentration, surfactant and homogenization time on the stability and particle size was also investigated. The Particle size and zeta potential (that measures the electro-kinetic potential of nano-suspensions) results showed that most stable β-carotene nanosuspension was obtained at 98% water concentration and 5% lecithin "based on emulsion mass" for 4 minutes emulsification. Also, the specified nanosuspension showed high physico-chmical stability.[32]

In 2006, Anantachoke et al tried to formulate β-carotene monodispersed nanoparticles using spinning disk processing (SDP), (where a very high centrifugal force is created under the effect of the spinning desk, and this force in the presence of high voltage will cause particle repulsion to yiels nanoparticles) under the electric shown in Figure 2-1. This creates very thin (1 to 200 µm) fluid layers on a rapidly spinning surface (10 to 3000 rpm), within which nano particulation takes place. Indeed, some surfactants were used to stabilize the nanoparticles at very low particle size. Dynamic light scattering results showed that the size of the particles varied with surfactant variation. However transmission electron microscopy (TEM) confirmed the spheroidal shape of the produced β-carotene nanoparticles. In brief, SDP technique can be used to produce organic nanoparticles that can be used for drug delivery systems and food fortification applications. [33]

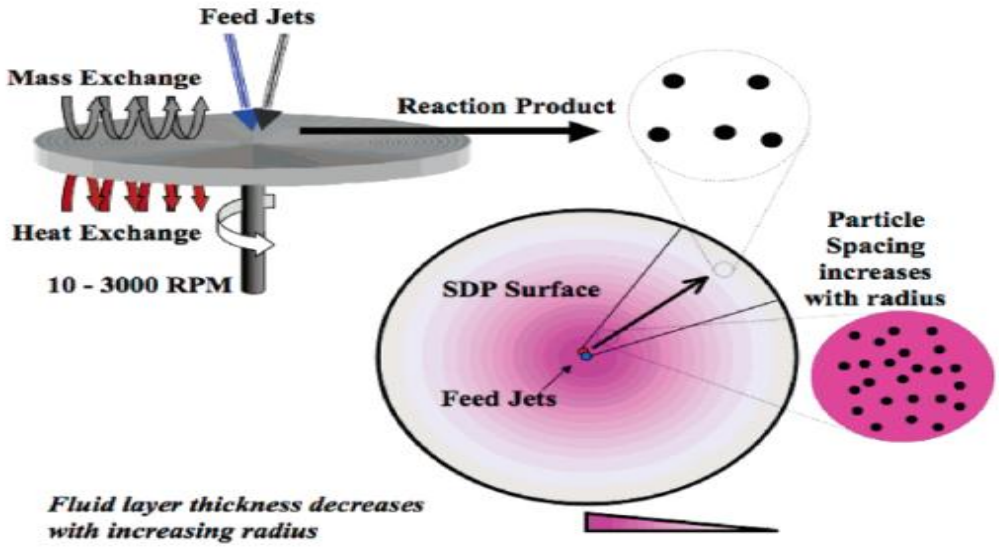


Figure 2-1. Schematic diagram for the SDP and the nucleation process that occurs on the disk.[33]

In 2012, Lacatusu et al. studied the use of squalene and grape seed oils as natural antioxidant oils to prepare nanostructured lipid carriers (NLCs) with the aim of protecting and stabilizing the sensitive β-carotene molecule.

Practically this was achieved by preparing several oil-in-water nano-emulsions stabilized by a mixture of surfactants at different high shear homogenization processes. The *in-vitro* results of β-carotene loaded NLC showed that the antioxidant activity of β-carotene after loading was enhanced and this was confirmed by the free oxygen quenching ability. Additionally, the prepared β-carotene-NLCs exhibited excellent physical stability with almost all zeta potential values. [34]

2.4. Zinc micronutrient in nanoparticles

Zinc oxide and other oxide nanomaterials were shown to have cytotoxic as well as anti-bacterial activity, thus, a simple way for ZnO nanoparticle synthesis from $ZnCl_2$ was proposed by Renugadevi et al. The size and composition of the produced nanoparticles were analyzed using Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDX). The antibacterial effect of ZnO was studied by the disc diffusion method against *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Salmonella typhi*, and *Vibrio cholera*. Moreover, the cytotoxic activity of the nanoparticles was examined against Vero cell line. In conclusion, the results confirmed the antibacterial activity of ZnO nanoparticles against all the previously mentioned strains of bacteria. However the cytotoxicity of ZnO nanoparticles was shown to be less than $ZnCl_2$ on Vero cell line.[35]

Raspopov et al. examined the effect of ZnO nano-particulation on the intestinal uptake and bioavailability of zinc on rats. The results showed that serum level of zinc was higher in rats treated with ZnO nanoparticles with respect to control samples (rats treated with normal Zn supplement). Also, it was confirmed that ZnO nanoparticles aided in the intestinal uptake of some macromolecules like egg albumin and other immune factors. [36]

2.5. Combining β-carotene with zinc for better pharmacological activity:

Combining zinc with β-carotene, as a pro-vitamin A molecule, enhances the therapeutic effects of the latter. In this respect, Dijkhuizen et al. examined the effect of iron and folic acid plus β-carotene or zinc or both during pregnancy to improve the micronutrient level of mothers and infants post-delivery. The results showed that vitamin A level was only improved when β-carotene was combined with zinc, as this aided in the conversion of more

β carotene molecules to vitamin A. However, the improvement of vitamin A was reflected on its increased content in breast milk and this consequently increased the vitamin A status of the infants. Indeed, adding zinc to β-carotene proved to be an efficient and cost-effective method to improve the healthiness of both lactating mothers and infants.[37]

In order to maximize the biological effect of β-carotene, Wieringa et al. tested the effect of zinc combined with β-carotene to treat micronutrient deficiencies in pregnant women. The findings revealed that maternal zinc and β-carotene supplementation enhanced the immunity functions in newborns, and such effective formula might be used in rural areas to decrease morbidity rate in infants in these areas. [38]

Chapter 3: Materials and methods

3.1. Materials

All materials were obtained and used without any extra purification. The following was used:

- Hexane (HPLC grade), Potassium bromide, Sodium phosphate monobasic di-hydrate (99%), Hydrochloric acid 37%, Dichloromethane (≥95%) and Beta carotene powder, Synthetic (≥ 93%) from Sigma Aldrich, UK.
- Bipro Whey protein isolate 92.6% (w/w) from Davisco Foods International Inc., Eden Prairie, USA.
- Calcium chloride, granulated, extra pure and Pepsin 1:3000 from Scharlau S.L., Spain.
- Ethanol absolute anhydrous from Carlo Erba Reagents, France.
- Sodium azide (≥ 98%) from Acros Organics, Belgium.
- Sodium hydroxide (≥ 98%) from Bio.chem, Egypt.
- Sodium phosphate dibasic obtained from Fine Chem, Egypt.
- Trypsin enzyme 2656.7 u/mg obtained from FAIZYME laboratories, Egypt.
- Zinc chloride (≥97%) from Laboratory Rasayan, India. HPLC water from Milli Q, Germany.
- PVDF filter of 0.45µm from Sigma Aldrich, UK.

3.2. Equipment

- pH meter (Accumet AR10) to adjust the pH of the formulation, centrifuge (Centrific) to separate the protein particles from the supernatant and Heratherm Oven, all obtained from Fischer scientific, UK.

- Sonicator (150HT) to facilitate the dissociation of large whey protein nanoparticle aggregates. And Stirrer (VWR) from ETL testing laboratories, INC., USA.
- Vortex (V1 plus) for sample mixing from Boeco, Germany.
- Recipocal shaking bath (Model 25) from Precision scientific Instruments, Inc., USA.
- Zeta sizer (Image 3-1) (Nano-ZS90) to determine particle size, from Malvern Instruments, UK.
- Field emission scanning electron microscope (FESEM) to study the surface morphology of the nanoparticles (Supra 55) operated at 23 kV from Leo Zeiss, Germany
- Fourier transform infrared spectrometer (FTIR) (Nicolet 380) to determine the structure of the particles as well as the original materials. Obtained from Thermo Fischer scientific Inc., UK.
- High Performance Liquid Chromatography (HPLC) (DIONEX Ultimate 3000 Series) equipped with Ultimate 3000 pump and Photo Diode Array detector to determine β-carotene concentration in the nanoparticle preparation as well as the control sample. Peaks were separated on a BDS HYPERSIL C18 column using a mobile phase system composed of acetonitrile and dichloromethane (3:1) (v/v). The HPLC was operated in isocratic mode at flow rate of 1.5 ml/min. The absorption of β-carotene was detected at 450 nm, as this was found to be the wavelength of maximum absorption after scanning it in the visible range (450 nm-600). 20 µl sample was injected automatically using Ultimate 3000 autosampler and Chromeleon was used for peak integration and calculation. HPLC obtained from Thermo Fischer scientific Inc. UK.

- Ultraviolet Visible (UV-Vis) spectrometer (Spectronic 20 D^{+}), using Tungesten lamp, to determine the concentration of β-carotene released, from Thermo Electron Corporation, UK.
- Atomic absorption (200 series) operating with Zinc lamp at λ 213.9 nm to determine the concentration of zinc ions incorporated in nanoparticles, from Agilent Technologies, USA.

3.3. Sample preparation

3.3.1. Preparation of whey protein soluble polymers

Whey protein isolate (WPI) dispersion of 8% (w/v) was prepared using deionized water and adjusted with 1N NaOH to pH 7.0. In order to prevent microbial growth, sodium azide was added at 0.02% (w/v). The dispersion was kept refrigerated at 4°C overnight for complete hydration, then it was warmed to room temperature and degassed for 20 minutes under vacuum (560 mm Hg). The dispersion was then warmed up in a water bath to 80°C at a heating rate of 8°C/min and kept for 15 minutes at this temperature. The preparation was then cooled to room temperature on an ice bath, and a dilution of 2% (v/v) from the above protein concentrate in deionized water was prepared.

3.3.2. Preparation of β carotene and zinc loaded nanoparticles

The nanoparticles were prepared by the pH-cycling method previously described by Giroux et al. [20] as it is the method described for incorporation of hydrophobic moieties in whey protein isolate nanoparticles, but after introducing some modifications. A volume of 4 ml of 0.25 mg/ml β carotene in absolute ethanol was added per 250 ml WPI preparation. The mixture was then acidified under stirring using 0.1N HCl to an aggregation pH of 6.0. To this mixture, 1 ml of 62.5 mg/ml $CaCl_2$ solution and 1 ml of 7.5 mg/ml $ZnCl_2$ solution were added to reach final concentration of 2.25 mM and 0.25 mM respectively in the final mixture. These concentrations were determined after trying different concentration`s ratios and found that the previously stated one is the ideal one in terms of turbidity of the produced preparation and uniformity of particle size.

The preparation was left at 4°C in the fridge for an aggregation period of 22 h to allow the di-sulphide bonds to form. The dispersion was then brought to pH 7 using 1N NaOH at room temperature to disrupt the non-covalent bonds and restore the electrostatic repulsions of aggregates. For further dissociation of nanoparticles aggregates; the dispersion was sonicated for 10 minutes at room temperature.

Part of the formula was kept in the liquid form for particle size, HPLC and atomic absorption analysis, while the rest of the formula (200 ml) was dried through the following procedure.

3.3.3. Sample drying

200 ml from the β-carotene and zinc loaded nanoparticle preparation was filtered through 3µm pore size filter paper. The precipitate was then washed using 20 ml hexane then the precipitate was collected in a porcelain dish, and allowed to dry under vacuum overnight. The large particles in the dried precipitate was then ground using a mortar and pestle. The particles were used then for FTIR analysis and *in-vitro* release study using UV spectrometer.

3.3.4. Preparation of blank sample

A blank sample of whey protein nanoparticles was prepared by the same method but without the addition of β-carotene and zinc. Where 250 ml WPI was acidified under stirring using 0.1N HCl to an aggregation pH of 6.0. To this mixture, 1.1 ml of 62.5 mg/ml $CaCl_2$ solution was added to reach final concentration of 2.5 mM in the final mixture. The preparation was left at 4°C in the fridge for an aggregation period of 22 h. the dispersion was then brought to pH 7 using 1N NaOH at room temperature and then sonicated for 10 minutes at room temperature.

3.3.5. Preparation of control sample

A control sample of β-carotene was prepared at the same time by adding same amount of β-carotene (.005g) in 4 ml ethanol and added drop wise per 250 ml deionized water under stirring at room temperature.

All samples (control sample and β-carotene and zinc loaded nanoparticles) were stored in closed volumetric flasks, covered with aluminum foil to be protected from light at stored room temperature.

3.4. Characterization of nanoparticles

3.4.1. Encapsulation efficiency:

The encapsulation efficiency for β-carotene in whey protein isolate nanoparticles was determined by measuring the amount of β-carotene lost in the filtrate after filtering the 200 ml loaded nanoparticle preparation; where 5 ml hexane were used to extract β-carotene from 5 ml filtrate. Also, the amount of β-carotene in the 20 mls hexane used to wash loaded nanoparticles was determined using pre-constructed calibration curve on UV at 448 nm. (Appendix)

$$Encapsulation\ efficiency = weight\ of\ the\ loaded\ drug / the\ weight\ of\ the\ input\ drug * 100$$

3.4.2. Particle size analysis

1 ml of deionized water added to 1 ml of the final preparation (either blank whey protein nanoparticles or β-carotene and zinc loaded nanoparticles) and filtered through a 0.45 µm PVDF syringe then used for particle size analysis by dynamic light scattering at room temperature using 90 degree scattering optics

3.4.3. Scanning electron microscope:

One ml of nanoparticle formulation was filtered through a 0.45µm membrane disc filter, the filtrate was then centrifuged at very low speed (i.e. 100 rpm) for 10 minutes, as the protein nanoparticles are so fragile that even very low shearing force may result in particle disruption. Then the supernatant was disregarded to remove residual salts and the precipitate was re-dispersed again in 1ml fresh de-ionized water. The dispersion was then allowed to dry on aluminum sheet under nitrogen gas at room temperature and used as it is without sputtering for obtaining Scanning Electron Microscope images.

3.4.4 FT-IR (Fourier Transform Infrared Spectrophotometer):

FTIR spectra were obtained for untreated whey protein powder, pure β-carotene powder and β-carotene and zinc loaded whey protein nanoparticles using the KBr (Potassium Bromide) pellet method. About 0.1 mg of sample was mixed with about 1 g of spectroscopic grade KBr and subjected to 1400 KPa hydraulic pressure to produce the pellets which were characterized using FTIR instrument.

3.5. Stability study for β-carotene loaded in nanoparticles for HPLC analysis

The total amount of the β-carotene retained in whey protein nanoparticles was determined according to the method proposed by Abbasi et al. but with some modifications. [24] It is a liquid extraction method, where 1 ml of HPLC grade water and 1 ml of absolute ethanol were mixed with 1 ml of the β-carotene and zinc loaded whey protein nanoparticles after 1, 2, 5, and 7 days from the preparation date, to extract the β-carotene remained in the whey protein nanoparticles at these intervals. The mixture was vortexed for 1 minute at room temperature. 1 ml of hexane (immiscible with water) was then used to extract β-carotene from the water ethanol mixture and vortexed again for 1 minute at room temperature. This was followed by centrifugation at 400 rpm for 2 min and then the organic upper solution was transferred to a falcon tube and allowed to evaporate under nitrogen gas. Subsequently, 1 ml of mixture of acetonitrile and dichloromethane (3:1) (v/v) was added to dissolve the β-carotene residues remained after evaporation and used for HPLC analysis. The concentrations of β-carotene in whey protein nanoparticles were measured against control sample also stored for 1, 2, 5 and 7 days.

3.6. *In vitro* release of β-carotene from whey protein nanoparticles:

In vitro release is performed in simulated intestinal/gastric fluid at 37°C under acidic and neutral conditions after drying the β-carotene and zinc loaded whey protein nanoparticles.

3.6.1. Preparation of simulated intestinal fluid

0.1 M phosphate buffer solution was prepared by dissolving 7.0g of NaH_2PO_4 and 21.8g Na_2HPO_4 in deionized water to make a total volume of 2 liters. The pH of the final solution was adjusted to 7.4 using 2N NaOH, and stored at 4°C. Finally, 2.0g of trypsin (intestinal enzyme) and 2.0g of sodium dodecyl sulfate, SDS, were added to the solution just before the analysis.

3.6.2. Preparation of simulated gastric fluid

A 2 liters of 0.1 M Phosphate buffer solution was adjusted to pH 1.6 with 2N HCl and stored at 4℃. Then 2.0g of pepsin enzyme was dissolved in the above buffer before the analysis at room temperature.

3.6.3. *In vitro* β-carotene release:

Both simulated fluids were incubated at 37°C in water bath for 1 hour before mixing with particles to be analyzed. The pre-weighed dried particles were divided into 15 portions and each portion was added to 20 ml simulated fluid in closed container. Each container was incubated at 37℃ in water bath with shaker at 200 shakes per minute at different times as specified in table 3-1 below;

Table 3-1. Dried particles in simulated fluids at different incubation periods.

Medium	Incubation time (min/hr)
Gastric simulated fluid	5 min
	10 min
	15 min
	30 min
	1 hr
	2 hr
Intestinal simulated fluid	1 hr
	2 hr
	4 hr
	6 hr
	8 hr

	12 hr
	16 hr
	20 hr
	24 hr

The released β-carotene in gastric fluid was solvent extracted with 20 ml Dichloromethane (DCM) and shaken for 2 minutes at room temperature then the lower layer was separated using a separating funnel. The absorbance was measured and the concentrations was determined using pre-constructed calibration curve at 460 nm.

In the case of intestinal fluid, the β-carotene released in the solution was measured directly, due to the presence of the emulsifier (SDS) which aid in solubilizing the released β-carotene molecules. The absorbance was measured at 407 nm and the concentration wad determined using pre-constructed calibration curve on UV (Appendix).

3.6. Zn^{+2} determination by atomic absorption;

Atomic absorption techniques was used to measure the amount of zinc that was successfully incorporated in β-carotene and zinc loaded whey protein nanoparticles and the amount that remained un-incorporated in the solution. In this respect, 1 ml of original β-carotene and zinc loaded whey protein nanoparticles diluted in 10 ml deionized water and vortexed for 1 min and left overnight at 70°C in closed vial to ensure complete degradation of protein nanoparticles, the solution was then used to determine total amount of zinc available in the solution (either free, adsorbed or incorporated in nanoparticles).

In another respect, 1 ml of β-carotene and zinc loaded whey protein nanoparticles preparation was filtered through 3 µm filter paper, the filtrate was then diluted with 10 ml deionized water and used for atomic absorption to determine the un-incorporated amount of zinc remained in the solution. However, the precipitate was then washed with 10 ml deionized water and used to measure the amount of zinc adsorbed on the nanoparticles without being incorporated in them.

However, all samples were used directly for Zn^{+2} concentration determination using calibration curve on atomic absorption spectrophotometer.

Chapter 4. Results and discussion

This chapter offers interpretation and discussion of the characterization, stability and release profile results for the zinc and β carotene loaded whey protein nanoparticles. These results were obtained through Zeta sizer, FTIR, SEM, HPLC, Atomic absorption and UV to quantify the amount of drug released in gastric and intestinal medium.

4.1. Encapsulation efficiency:

97% from the total amount of β-carotene added were successfully encapsulated in whey protein nanoparticles.

4.2. Particle size analysis

Table 4-1. summarizes the dynamic light scattering results of zinc and β-carotene loaded whey protein nanoparticles compared to the blank particles. The polydispersity index (PdI) for both samples was determined to be in an acceptable range (0.0-0.7) implying that the particle size distribution values for both samples is not too high and the result quality according to these values is acceptable.

However, there is a substantial difference between the particle sizes of whey protein nanoparticles and β-carotene loaded nanoparticles as shown in Table 4-1. The Z-average of whey protein nanoparticles was determined to be 96.1 nm, while the Z-average of β-carotene loaded nanoparticles was shown to be 409.7 nm. These results were confirmed by the statistical data presented in Figure 4-1, where the average size of the whey protein nanoparticles is around 71 nm while that of the β-carotene and zinc loaded whey protein nanoparticles is 400 nm. Such differences between these average sizes strongly suggest that β-carotene and zinc were successfully incorporated in the whey protein nanoparticles. It is thus not surprising that all the average sizes (Z-average, particle size and peak size distribution by volume) results of this formula were significantly larger than those of the blank samples (i.e. whey protein nanoparticles) although both formulations were prepared by the same method (pH cycle) and under the same conditions (aggregation pH 6 and ageing time 22 hr).

There is an important observation to note regarding Z-average in table 4-1 and the average size distribution by volume in figure 4-1. The Z-average value of the blank whey protein nanoparticles was found to be 96.01 nm, however Figure 4-1 shows that the average size, based on volume, for these particles is around 71 nm. . Presence of large aggregates may increase the overall Z-average value, however, this is confirmed by the presence of a very small peak at the right side of figure 4-1. Lovaley et al confirmed that , whey protein particles, by time, tend to aggregate and form large particles, due to hydrophobic and electrostatic interactions [39] Moreover, results obtained from DLS analysis are consistent with that obtained by Giroux et al, as Z-average of whey protein nanoparticles prepared at pH 6 and 22hr ageing time was 100 nm.[20] Same explanation could be applied to the results of formulated zinc and β- carotene loaded nanoparticles, as some particles may aggregate to shift the Z-average to higher value. This was confirmed by the presence of a very small peak at the right side of the graph 4-1.

Table 4-1. DLS results

Parameter	*Whey protein nanoparticle*	*Zn and β-carotene loaded nanoparticles*
PdI (poly dispersity index)	*0.346*	*0.269*
Z-average (d.nm)	*96.19*	*409.7*

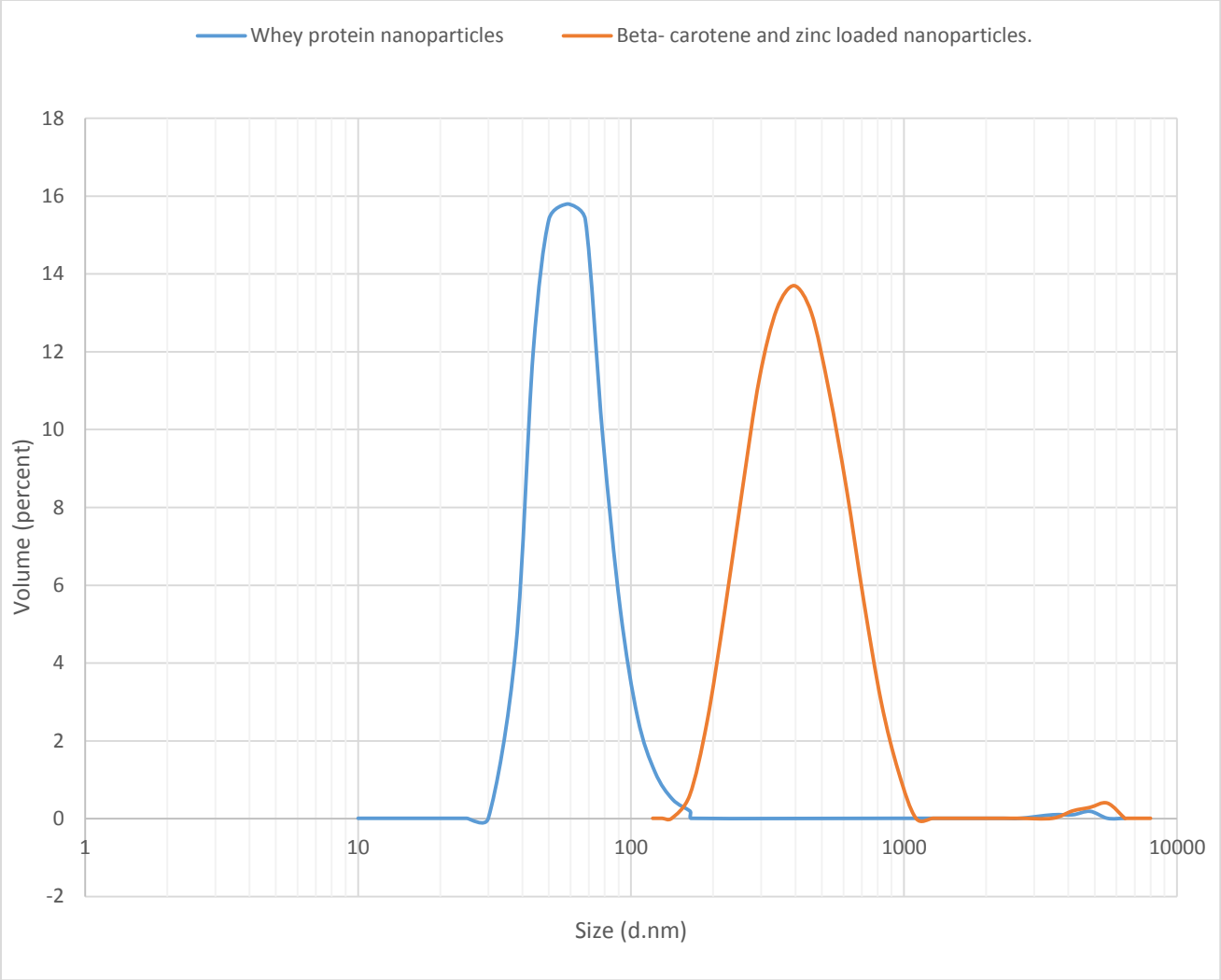


Figure 4-1. Size distribution by volume for whey protein nanoparticles and β-carotene loaded whey protein nanoparticles.

4.3. SEM

SEM images for whey protein nanoparticles as well as zinc and β-carotene loaded nanoparticles are shown in Figures 4-2, 4-3, 4-4 and 4-5, 4-6,4-7 respectively. It is clear from the images shown in Figure 4-7 and 4-8 that whey protein nanoparticles have retained their spherical structure after zinc and β-carotene encapsulation. Also both samples from a 2µm scale shows leaf like structure as in Figures 4-2 and 4-5. The major difference being the size of the particles, as the average size for zinc and β-carotene loaded whey protein nanoparticles from a 200 nm sclae(as in figure 4-7) is estimated to be larger than the blank whey protein nanoparticles (figure 4-3). The whey protein nanoparticles in both samples tended to form large aggregates most likely due to the presence of charged species (i.e. Ca^{2+}, Zn^{2+} and charged whey protein nanoparticles) and also due to hydrophobic interactions [39]. Same spherical shape of blank whey protein nanoparticles, were obtained by Gulseren *et al*, although the method of preparation was different, as they used ethanol desolvation method to prepare whey protein nanoparticles. [25]

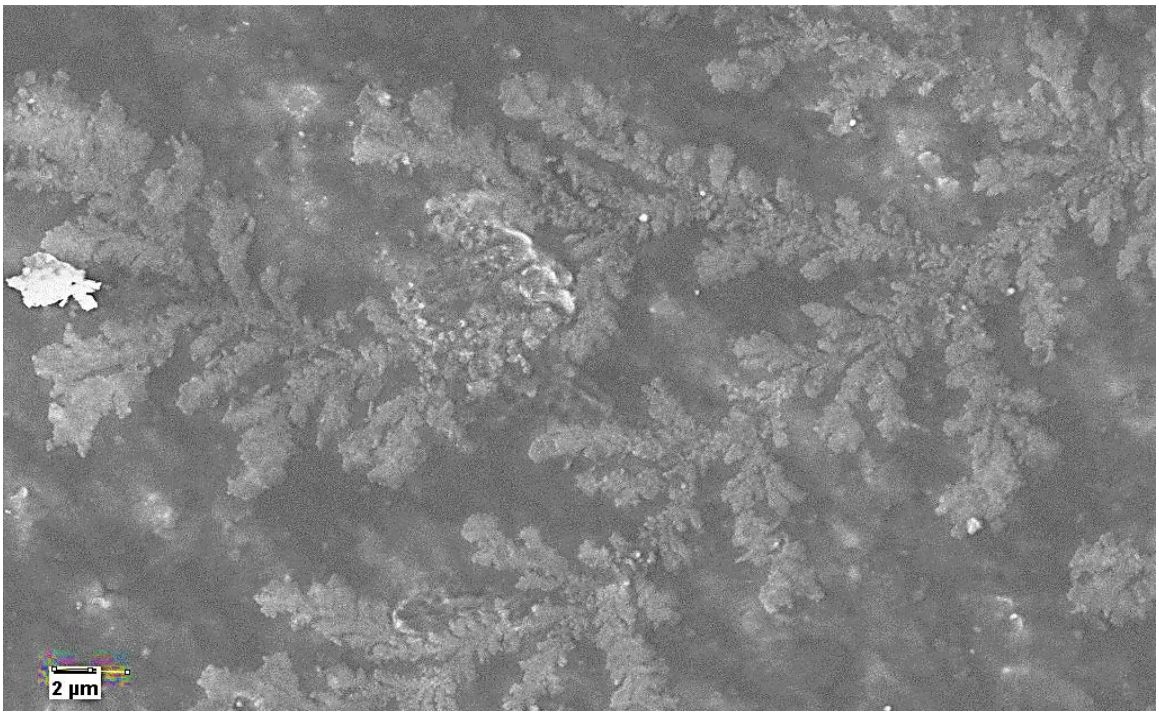


Figure 4-2. SEM image for whey protein nanoparticles (Blank) from 2 µm scale

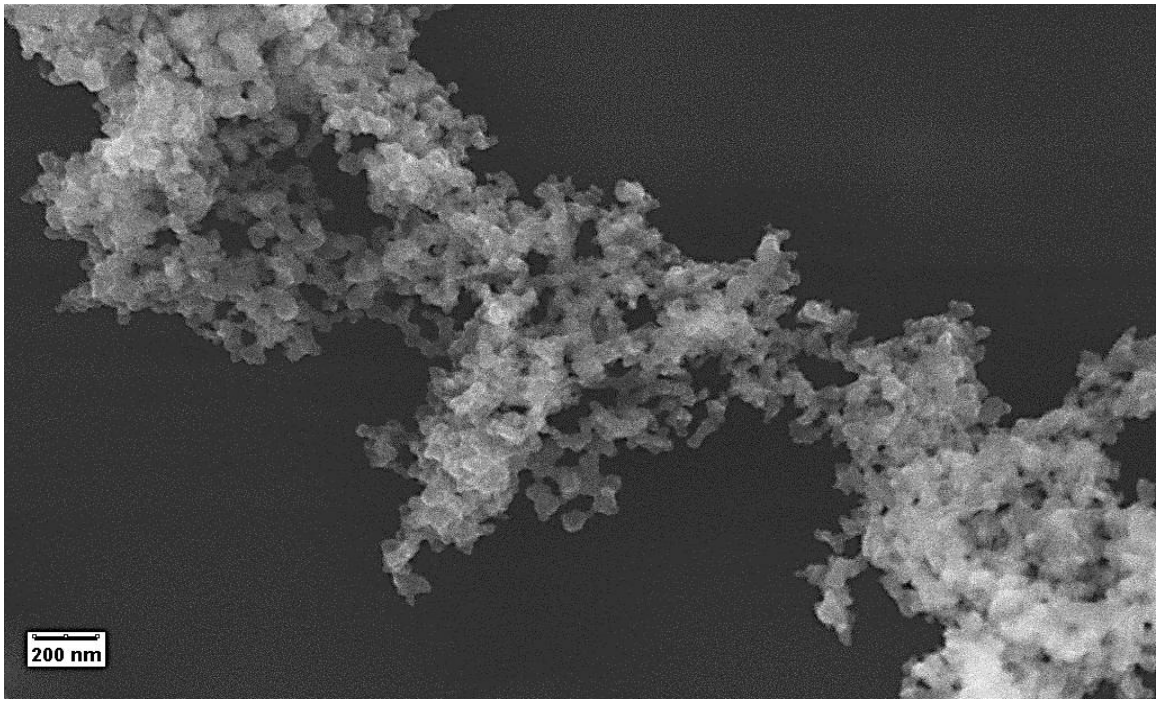


Figure 4-3. SEM image of whey protein nanoparticles from 200 nm scale

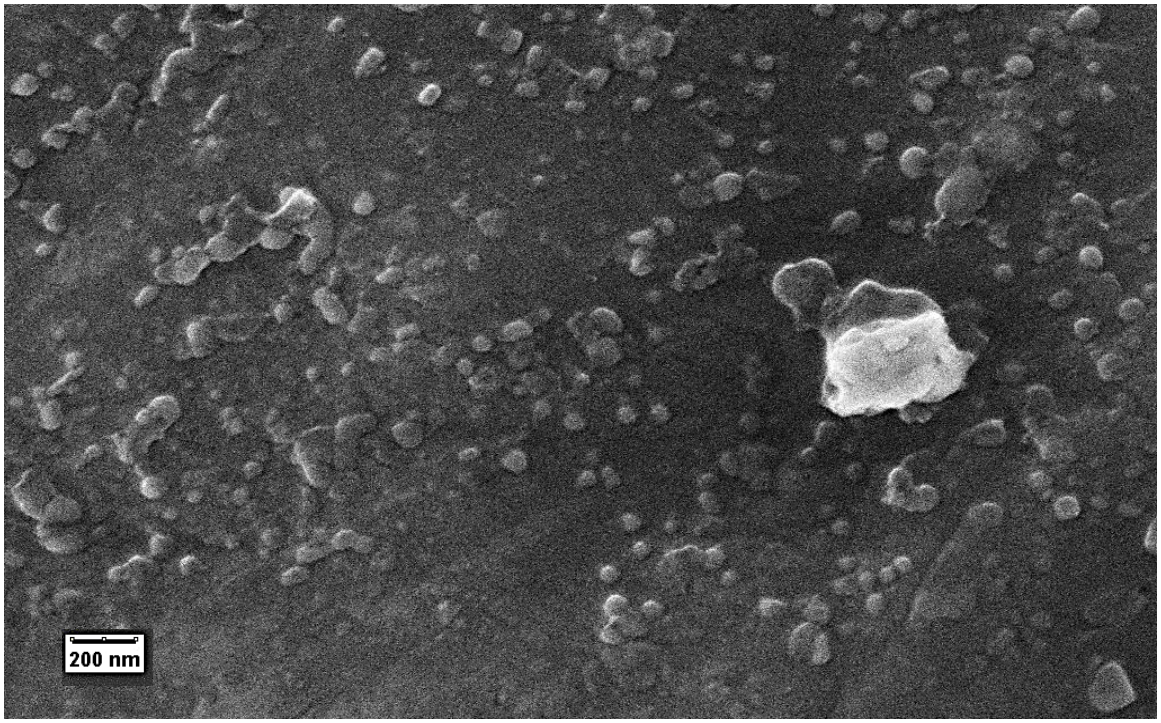


Figure 4-4. SEM images for whey protein nanoparticles from 200 nm scale (higher magnification)

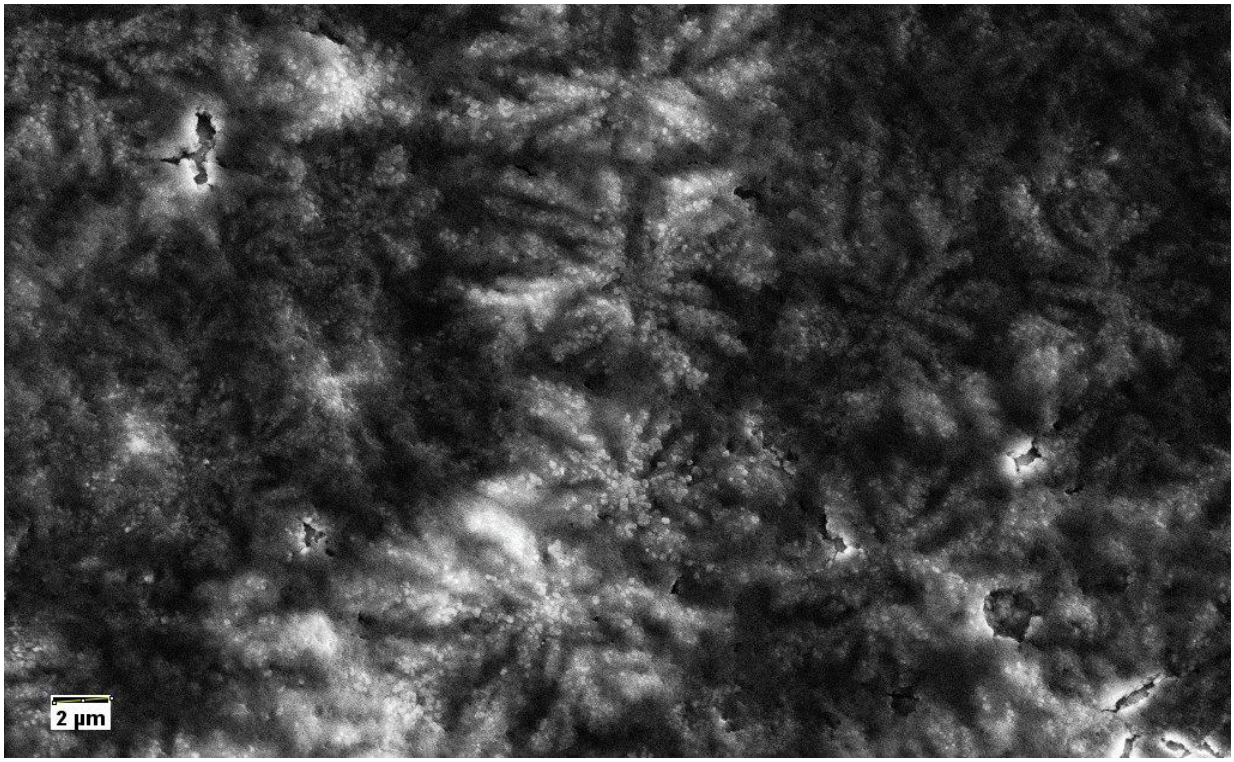


Figure 4-6. SEM image for β-carotene and zinc loaded whey protein nanoparticles from 2µm scale

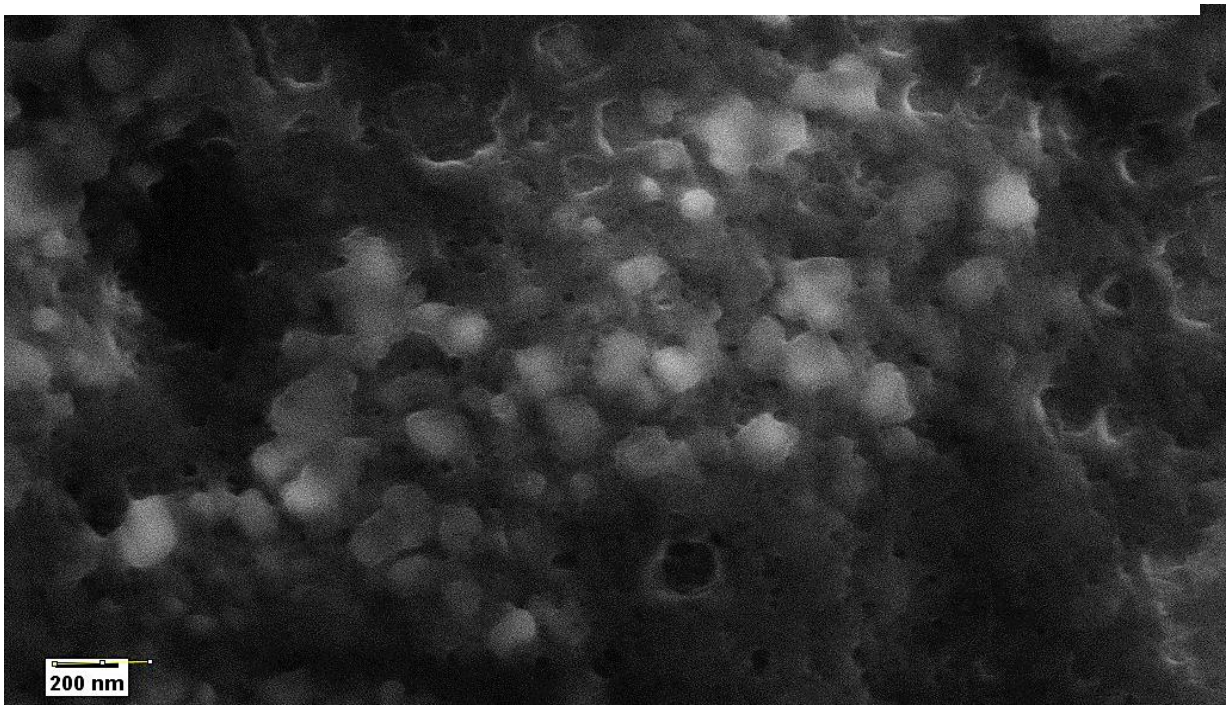


Figure 4-8. SEM image for β-carotene and zinc loaded whey protein nanoparticles on a 200 nm scale

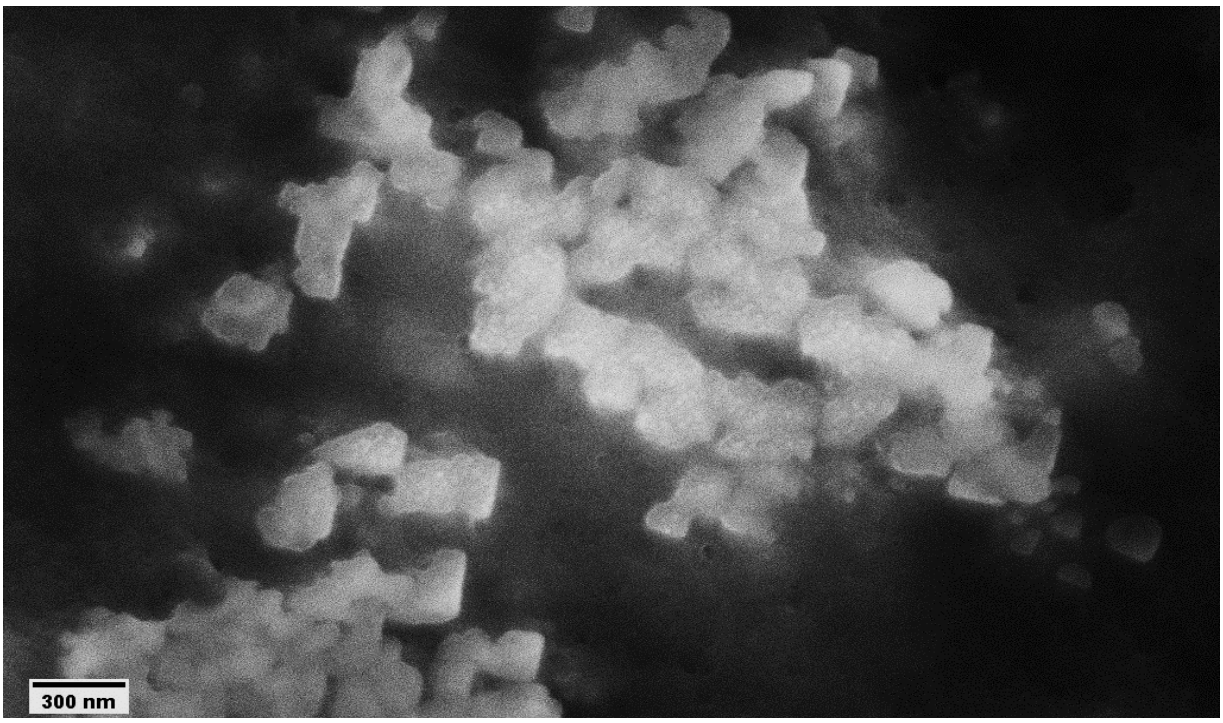


Figure 4-7. SEM image for β-carotene and zinc loaded whey protein nanoparticles from a 300 nm magnification scale.

4.4. FT-IR:

FT-IR characterization was conducted to determine whether the β-carotene had been successfully encapsulated in whey protein nanoparticles, as well as to determine the type of interactions that took place during the encapsulation process. The FTIR spectra of untreated whey protein, β-carotene as well as the zinc and β-carotene loaded nanoparticles are shown in the figure below (Fig. 4-8).

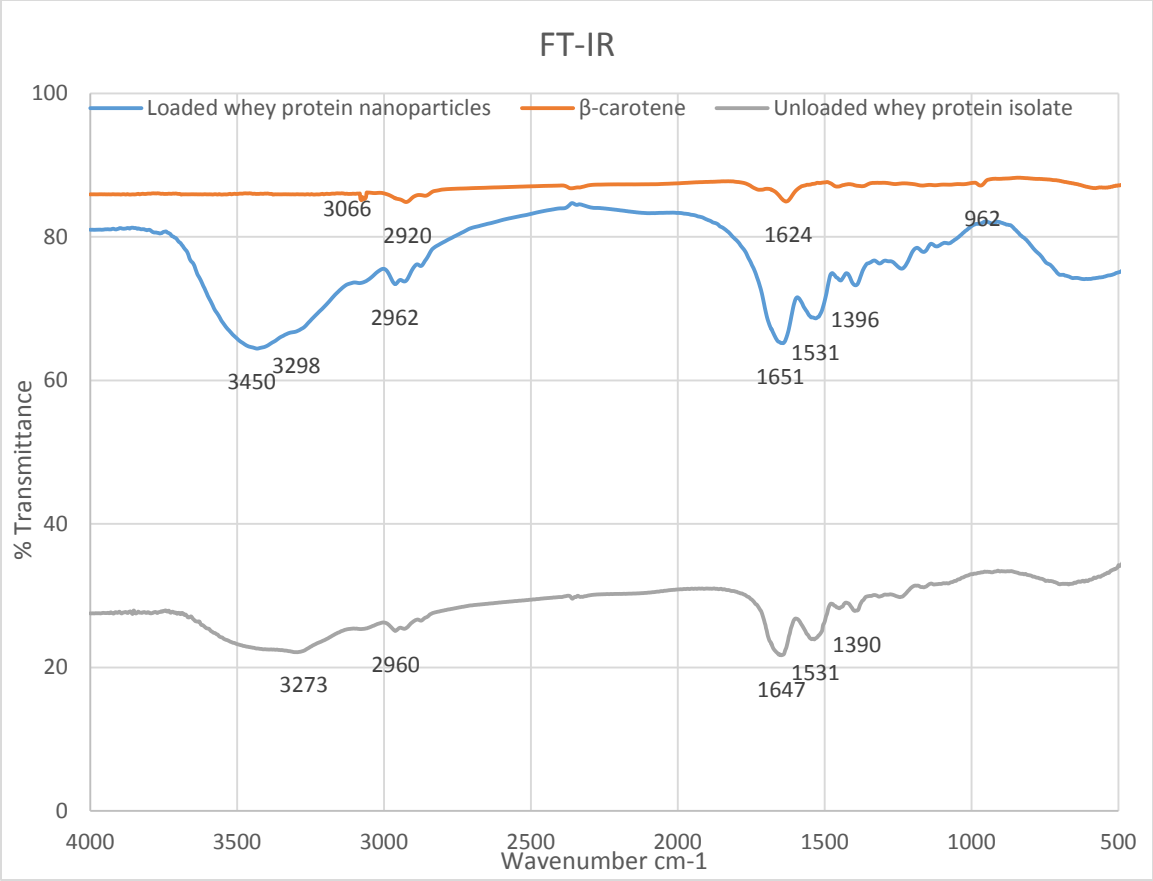


Figure 4-8. FTIR of pure β-carotene (Upper trace), zinc and β-carotene loaded whey protein nanoparticles (trace in the middle) and unloaded whey protein (lower trace).

The upper trace represents the IR spectrum of β-carotene alone, where the peak of the C-H stretches vibrations in the alkyl group that is attached to the cyclohexene end group in β-carotene is seen at 2919 cm^{-1}, however, the C-H aromatic appears on the left of C-H aliphatic at 3066 cm^{-1}. The very characteristic peak for the C=C stretches exist in the two terminal cyclohexene groups appears at 1625 cm^{-1}, yet, the C-C stretches vibrations in cycle hexane ring is seen at 1457 cm^{-1} .[40] Another characteristic peak

of β-carotene molecule appears at 962 cm^{-1}, which is characteristic for the C-H of conjugated alkene. [28]

The bottom trace of Figure 4-8 shows the IR spectrum of un-treated whey protein which consists mainly of α-lactalbumin and β-lactoglobulin along with minor proteins like bovine serum albumin and immunoglobulin G. The IR spectrum shows a very large peak at 3272 cm^{-1} which is very characteristic to the hydroxyl (OH) group that is present in many amino acids that build up the structure of α-lactalbumin and β-lactoglobulin, (the most abundant protein units in whey protein), these amino acids are Tyrosine, Threonine and Serine (Figure 4-9). [28]

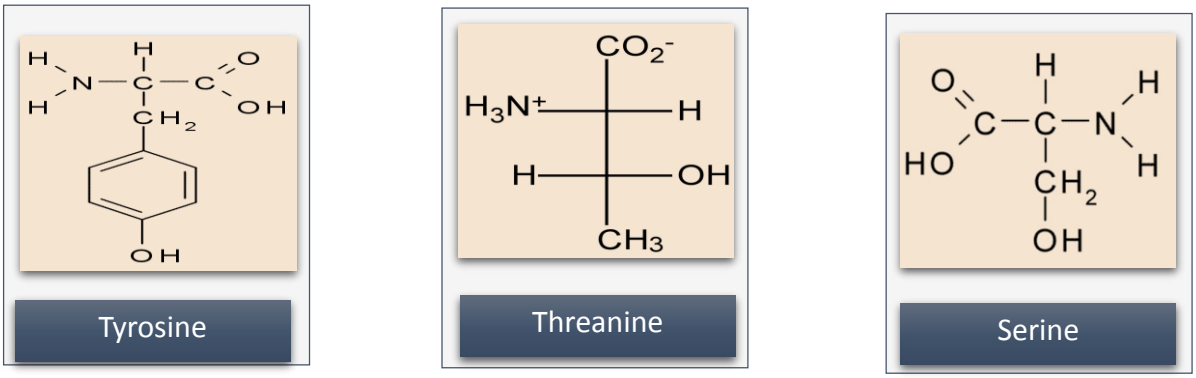


Figure 4-9 Amino acids with (OH) polar groups [43]

The very broad peak for the phenolic O-H stretch bond appears at 3450 cm^{-1}, while the peak for the carboxylic O-H stretch vibration appears at 2960 cm^{-1}, however there is an overlap between the phenolic OH and the amine (N-H) stretching peaks seen at 2434 cm^{-1}, as both signals appear at almost the same wavenumber range. [40] Every amino acid contains one amine group but some like Tryptophan, Glutamine, Asparagine and Methionine have extra amine groups (Figure 4-10).

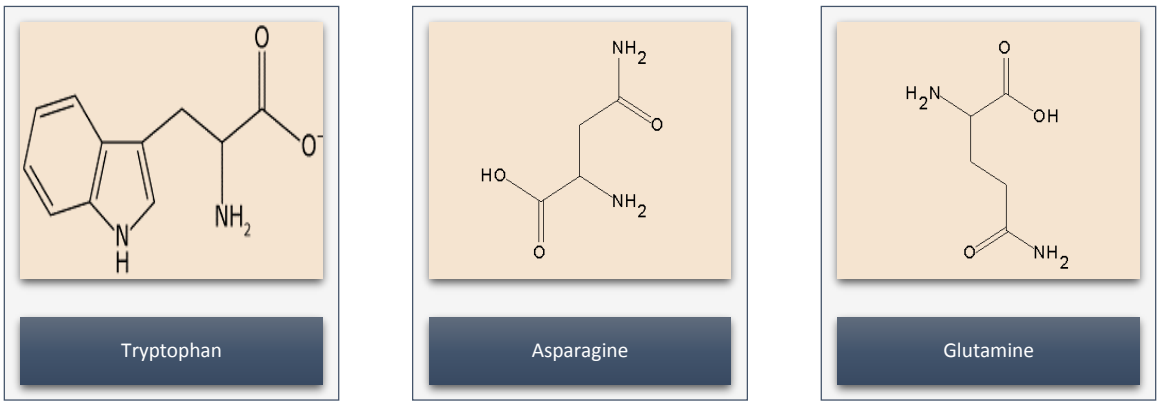


Figure 4-10. Amino acids with More than one amine group.[39]

There is a significant peak at 1646 cm^{-1} corresponding to the amide C=O stretch vibration that links the amino acids together to form the peptide. Moreover, the C=C aromatic bending vibrations appeared at a characteristic wavenumber range from 1500-1700 cm^{-1}. [41]

The middle trace in Figure 4-8 represents the IR spectrum of zinc and β-carotene loaded nanoparticles. The characteristic peaks for β-carotene and the whey protein were retained. Some shifts in the frequency of bands can be observed for the nanoparticle with the β-carotene. The O-H, N-H stretching peaks appeared with a very slight shift to lower frequencies. This may be due to a possible attraction between the amino acids and Ca^{+2} or Zn^{+2} ions added during the preparation of the drug loaded nanoparticles. Calcium ions can interact with the proteins through sites such as OH and NH of the nanoparticles after protein denaturation. This shift could also be attributed to possible hydrogen bonding occurring between some functional groups of the amino acid. Also the typical C=O of the whey protein as well as the C=C had appeared at a higher wavenumbers suggesting possible interactions with the metal ions. Thus it is concluded that adding β-carotene and Zinc Chloride to the whey protein suspension near the isoelectric point of the protein (which is at pH 6) had resulted in a very minimal changes in the IR

spectra of both materials suggesting that the mechanism of encapsulation involved mainly hydrophobic or physical interactions. The addition of calcium chloride and zinc chloride at the same pH to neutralize the electrostatic charges of protein followed by letting the formula to age for ~22 hr (to allow the disulfide cross linking between protein strands to take place) also had resulted in formation of stable nanoparticles.

4.5. HPLC analysis:

HPLC analysis was carried out to examine the stabilizing and protecting effect of whey protein nanoparticles on β-carotene, which is known to be heat, light, and pH sensitive. Thus, HPLC testing for β-carotene loaded nanoparticles was conducted against control samples

The HPLC results presented below (Figure 4-11) shows that at day 1 the amount of β-carotene retained in the nanoparticles was virtually the same as in the control sample. The retention of β-carotene however was shown to be significantly higher in nanoparticles at 2, 5 and 7 day time points relative to the control.

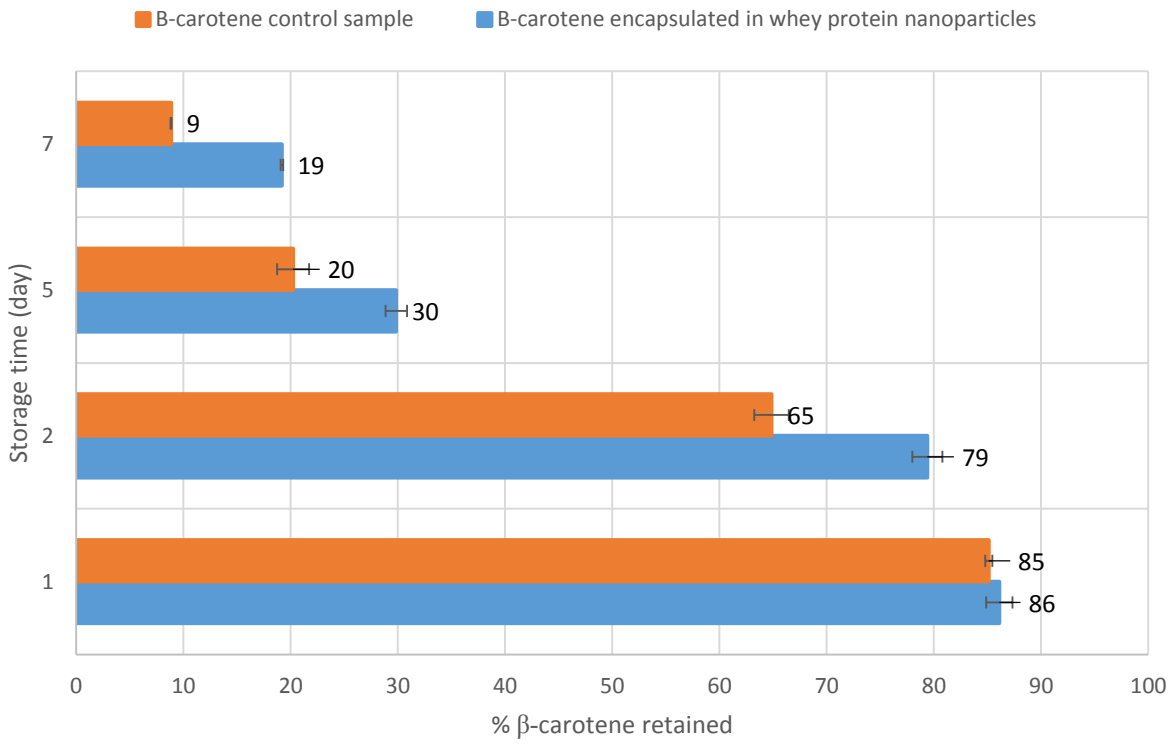
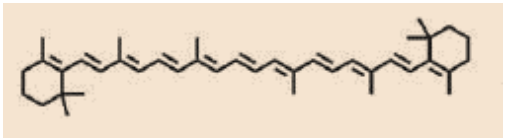


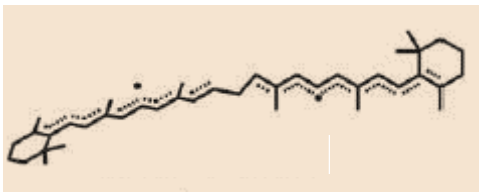
Figure 4-11. Percentage of β-carotene retained at different storage intervals

β-carotene is very susceptible to oxidation reactions under stressing conditions, like light, heat and reactive oxygen species. Figure 4-12 illustrates the structures of β-carotene oxidation products resulting from reactions with reactive oxygen species (ROS). The process of β-carotene oxidation is an autocatalytic reaction, and this may explain the large drop (74% drop in case of test sample and 67% drop in case of loaded nanoparticles at day 7) in β-carotene concentration that took place in the control sample when compared to nanoparticle preparation, although both samples were kept in the dark at room temperature. The initiation of the autoxidation reaction was explained extensively by Boon *et al*. [42] where they proved that the initiation of the autoxidation happens when β-carotene goes through an isomerization step (which can occur easily even at 30̊ C in a β-carotene solution) to form a bi-radical "twisted" molecule β-carotene bi-radical (Figure 4-12). The twisting of this molecule leads to an unpaired

spin state, which makes it vulnerable to interactions with oxygen to form β-carotene epoxides and endoperoxides.

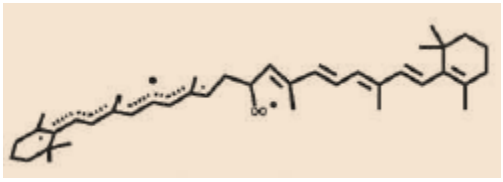


β-carotene

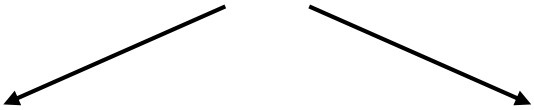


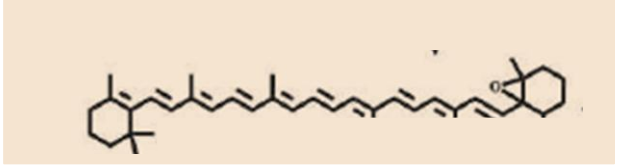
β-carotene bi-radical

O_2

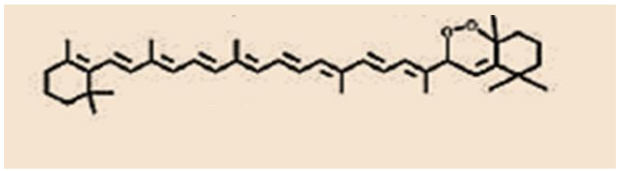


Carbon-peroxyl triplet bi-radical





5,6-Epoxide



Endoperoxides

Figure 4-12. Structures of Auto oxidation by- products of β-carotene [14]

On the other hand, β-carotene incorporated in whey protein nanoparticles showed higher stability profiles with respect to the control samples, as the incorporation process had limited the access of oxygen molecules to the entrapped β-carotene molecules leading to higher protection, hence, higher stability, and this was confirmed by the HPLC results shown in Figure 4-11.

These finding were very close to that obtained by Abbasi et al , who studied the effect of vitamin D encapsulation in whey protein nanoparticles, Vitamin D_3 is also susceptible to auto-oxidation reactions in the presence of light and air due to its conjugated triene structure (Figure 4-13). Abbasi et al confirmed that encapsulation of vitamin D_3 in whey prtotein nanoparticles had increased its stability in the presence of air when compared with a control sample (Vitamin D_3 in water-ethanol mixture) for 7 days, as the % of vitamin D_3 retained after 7 days was almost double the amount retained in the control sample . [24]

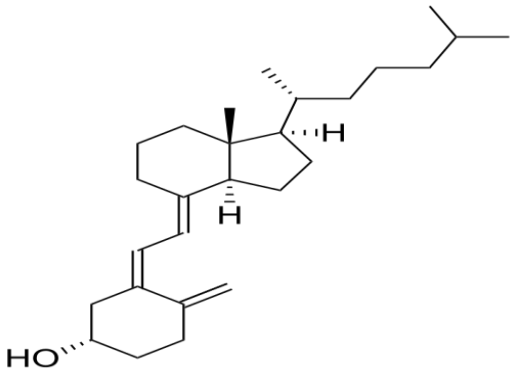


Figure 4-14. Vitamin D_3 [22]

4.6. *In-vitro* drug release:

UV analysis

In-vitro release studies were performed on the zinc and β-carotene loaded nanoparticles in gastric and intestinal mediums. Figures 4-15 and 4-16 summarize the release profile of the sample in both mediums at different time intervals.

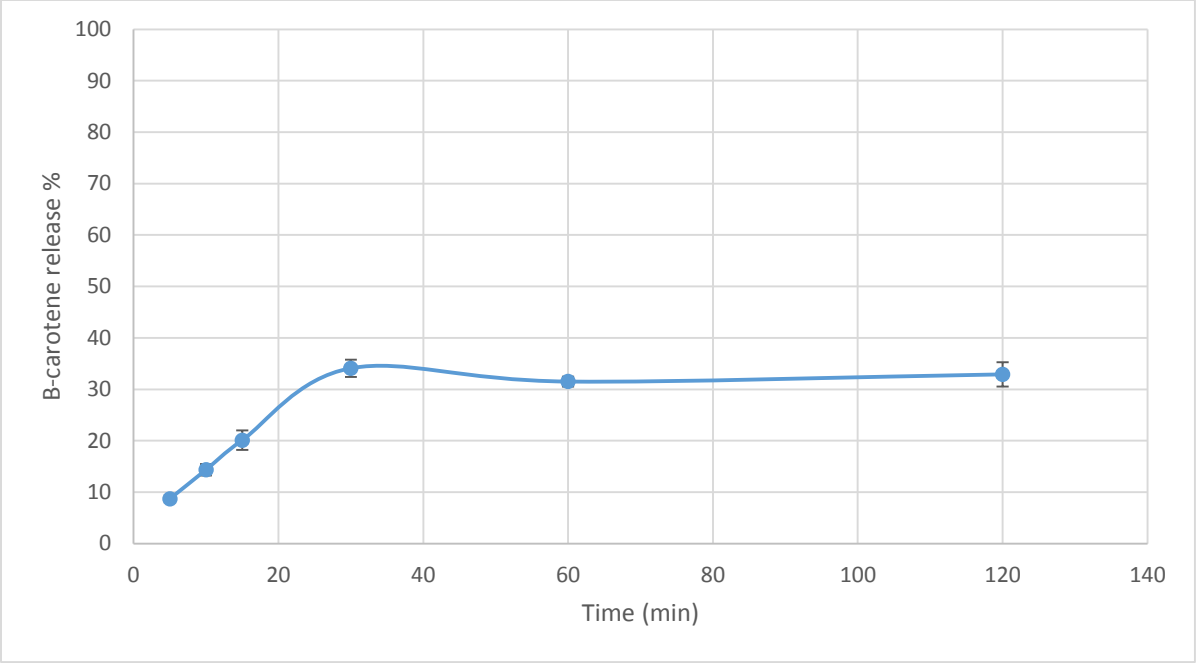


Figure 4-15. β-carotene release profile in gastric fluid from B-carotene and zinc loaded nanoparticles

Figure 4-15 shows the amount of β-carotene releases from zinc and β-carotene loaded nanoparticles in gastric simulated fluid at pH 2. It is clear from the figure that about 33 % from total β-carotene is released in the gastric fluid after 2 hours. This can be attributed to the presence of pepsin enzymes, which hydrolyze the peptide bonds between amino acids thus releasing the β-carotene. It is also important to note that subjecting whey protein nanoparticles to very low pH values (~2) may result in significant electrostatic repulsion between protein strands which may subsequently lead to dissociation and disruption of the protein structure making up the nanoparticles.

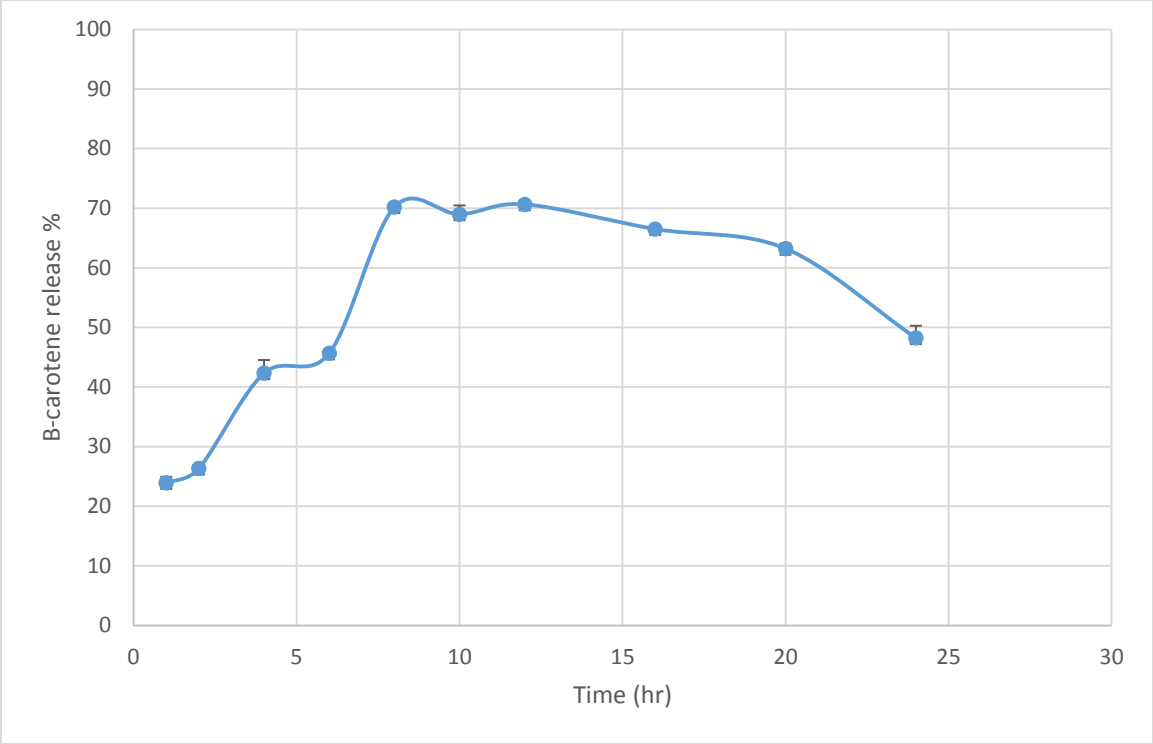


Figure 4-16. β-carotene release profile from B-carotene and zinc whey protein nanoparticles in intestinal fluid.

Figure 14-16 shows the release profile in intestinal fluid. However, β-carotene released at the first two hours was around 30%, reaching a maximum after 8 hours (70%) then moving to a plateau till 16 hrs. Starting 16 hrs, the drug started to degrade because as mentioned earlier in the HPLC results, the β-carotene moiety is very susceptible to autoxidation reactions that will lead to formation of biologically ineffective by-products, this explains the drop in Beta-carotene concentration found in intestinal fluid after 16 hrs. The presence of trypsin enzymes in the simulated intestinal fluid most likely led to the denaturing of the whey protein nanoparticles and the subsequent β-carotene release. Interestingly, the amount released after 2 hours in simulated gastric fluid was almost the same as that in the intestinal fluid (~30 %), which suggests that the main factor that controls β-carotene release is the presence of proteo-lytic enzymes (either Pepsin in gastric fluid or Trypsin in intestinal fluid). In the intestinal fluid, maximum release was observed after 8 hours where 70 % of loaded β-carotene was released, but

the maximum release (around 33%) in gastric fluid was observed after 30 minutes.

The change in pH, on the other hand, (from ~ 2 in the simulated gastric fluid to 7.4 in the simulated intestinal fluid) was shown not to affect the drug release profile for the drug at least for the first two hours.

Almost the same release profile results were obtained by Jiang et al, which confirm that whey protein isolate nanoparticles are good carriers to deliver β-carotene as they exhibit low release profiles in gastric fluids and high release profile in intestinal fluids, although they prepared the whey protein nanoparticles by different method than those used here. [28]

These results are of great advantage as the main site for β-carotene absorption in humans is in intestine through the intestinal mucosa, thus being able to survive harsh gastric conditions (i.e. very low pH in the presence of pepsin) and also being able deliver up to 70% of total β-carotene loaded in the nanoparticles to this site means that whey-protein nanoparticles can work as a good delivery vehicle for β-carotene. This conclusion is confirmed by the result of the Encapsulation efficiency which shows that 97.8 % of drug added was successfully trapped in the nanoparticles.

4.7. Atomic absorption

Atomic absorption measurements, performed to assess the amount of zinc that was able to bind to the negatively charged groups in whey protein nanoparticles during the pH cycle, and it showed that 33% of the zinc ions were attached to the nanoparticles. Such a low binding percentage may be explained by the presence of significantly higher calcium concentration ~2.25 mM (which part of it will participate in the formation of whey protein nanoparticles), relative to that of Zn which is 0.25 mM, and both ions will compete to bind to the negatively charged groups in protein amino acids, the Moreover, a research conducted by Eslam *et al*, had confirmed that the binding energy of calcium to di –peptide proteins like carnosine ($\Delta H°_{298}$= -187) is higher than that of Zinc ($\Delta H°_{298}$= -104) in solution phase [43], and as long as the main protein present in whey is also a di-peptide protein (β-

lactoglobulin), this may explain the low binding percentage of Zinc to whey protein particles.

Chapter 5. Conclusion and future work:

5.1. Conclusion

Whey which is the liquid by-product produced during cheese-making is recognized nowadays as one of the most nutritious and valuable milk components. Whey protein isolate (WPI) is derived from whey through extensive purified process, and it contains biologically active proteins (i.e. alpha-lactalbumin (ALA), beta-lactoglobulin (BLG), bovine serum albumin (BSA), and immune-globulins (IgG)), that can act as immune-modulator and antibacterial agent. Whey protein exerts superior emulsification and gelling properties, as β- lactoglobulin (most abundant protein in WPI) which a very effective gelling property it can be used as pH-sensitive hydrogels for the controlled delivery of biologically-active substances. Thus, whey protein isolate is used to make nanoparticles to encapsulate sensitive bio-active ingredients like β-carotene to protect it and decrease its rate of auto-oxidation reactions. β- carotene is an unsaturated hydrocarbon that shows a very potent anti-oxidant activity, by quenching singlet molecular oxygen and free radicals. Thus, β-carotene can reduce the risk of different diseases that are related to cells damage such as cancer, cardiovascular diseases, photosensitivity and aged-linked disorders. However, Zinc metal ion is classified as a potent antioxidant and also it has a role in β-carotene (pro-vitamin A) metabolism, including its absorption, transport, and utilization. It is therefore recommended to combine zinc ions with β-carotene supplementation.

Whey protein nanoparticles incorporating β-carotene and zinc is thus formulated to be used in food formulation as well as pharmaceutical preparation. These loaded nanoparticles were prepared by pH cycle method at aggregation pH 6 and 22 hours ageing time. The particles were then characterized using zeta sizer, SEM, FT-IR techniques. In addition, the stability of the loaded β-carotene and the release behavior at acidic and

neutral conditions were assessed using HPLC and UV spectrometer. Amount of zinc ions loaded also were calculated using atomic absorption.

Indeed, the results showed that around 97 % of β-carotene and 33% of zinc ions originally added to the formulation were successfully encapsulated in the whey protein nanoparticles, and 70% of the loaded β-carotene were released in the intestinal fluid. Also, the release study revealed that whey protein nanoparticles are hydrolyzed only in the presence proteo-lytic enzymes (pepsin and trypsin), as changing the pH didn't affect the release performance of whey protein nanoparticles. However, the stability of the β-carotene loaded in nanoparticles was higher than the control sample (β-carotene in water-ethanol mixture) after storage at same conditions for 7 days.

The SEM images have confirmed that the formulated nanoparticles are spherical in shape while the zeta sizer have showed that they are of around 400 nm in diameter. In general, the results obtained recommend usage of whey protein nanoparticles for encapsulation and drug delivery purposes in food and pharmaceutical industries.

5.2. Future work:

Future work should focus on the following;

- *In-vitro* antioxidant activity evaluation for the released beta-carotene.
- The use of whey protein nanoparticles for encapsulation of other hydrophobic nutritive ingredients, as this will hopefully allow their incorporation in any food and/or pharmaceutical formulation with acceptable release properties.
- Studying the effect of adding other metal ions on the aggregation behavior of whey protein nanoparticles.
- Sensory evaluation for the loaded whey protein nanoparticles to ensure that they will not have any impact on the sensory attributes of their final application.

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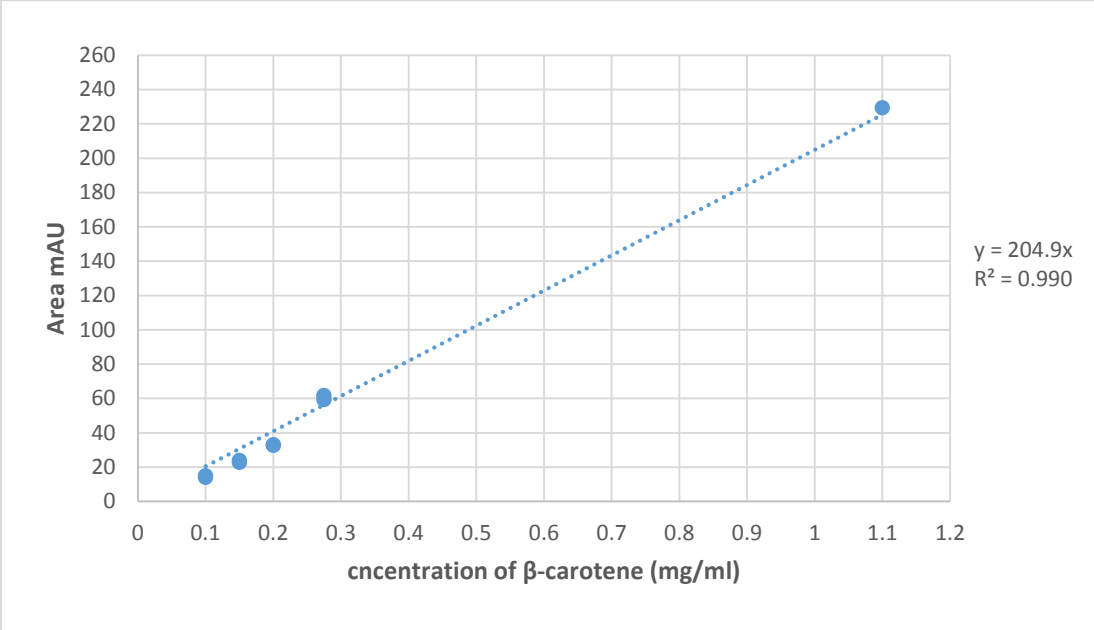
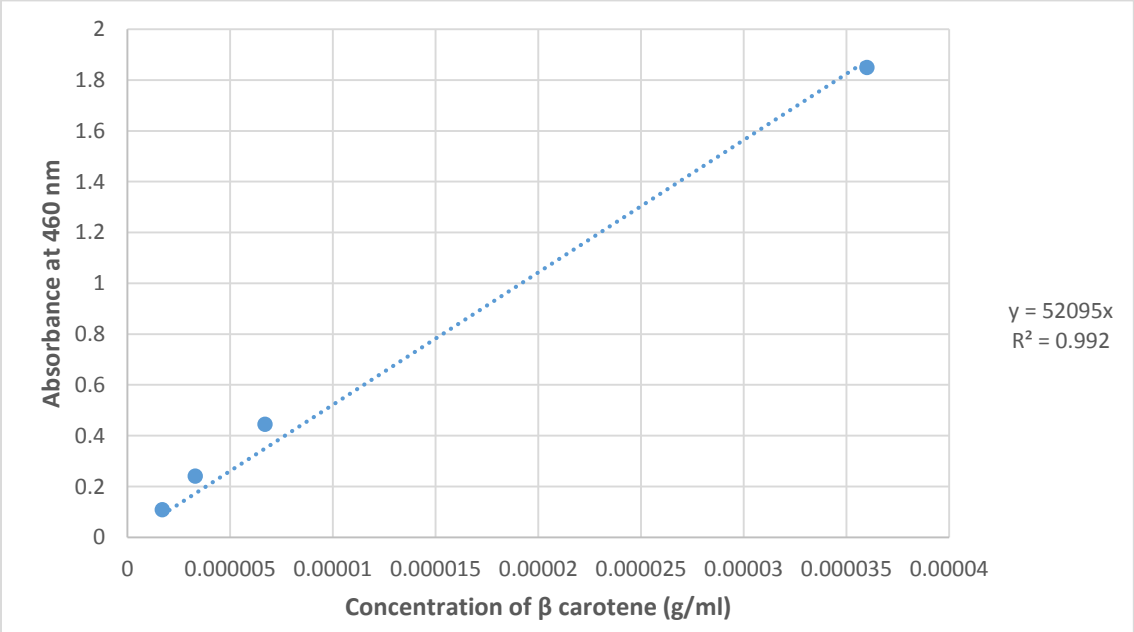
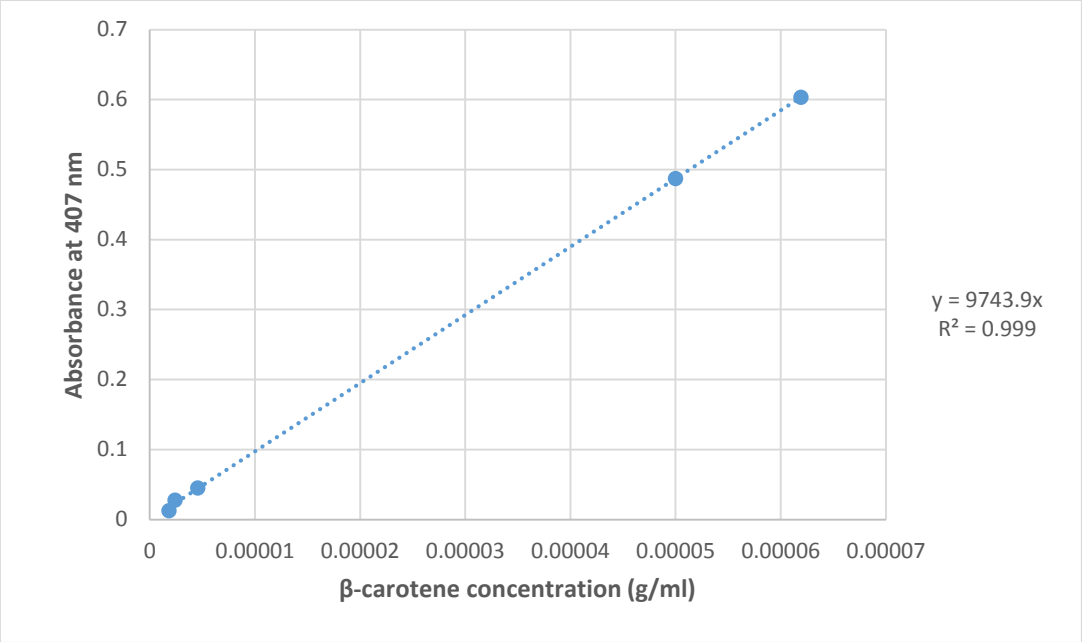
Appendix : Calibration curves



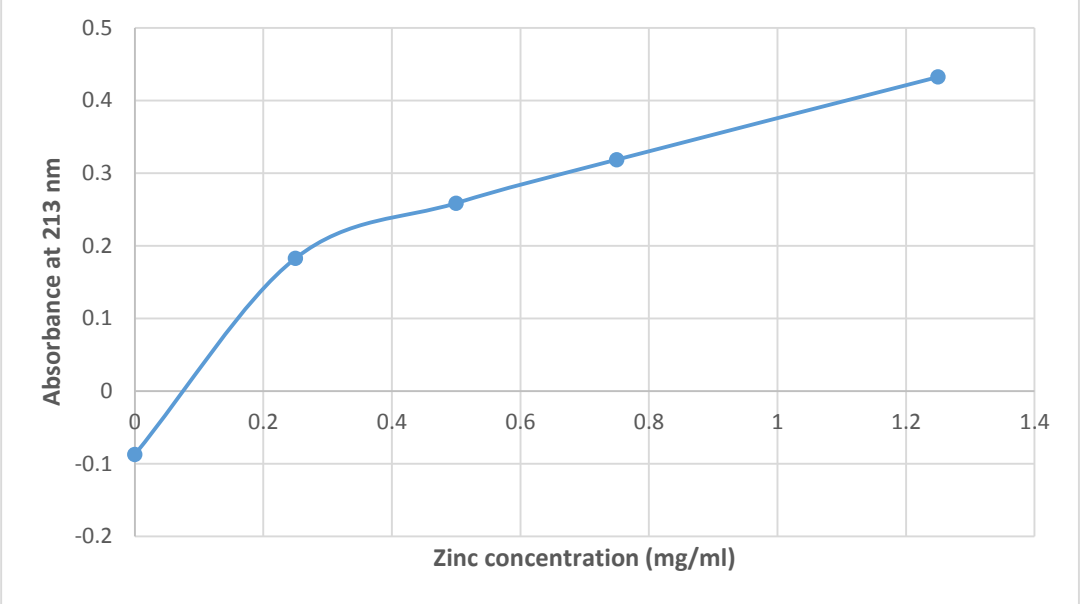
Calibration curve of β carotene (HPLC)



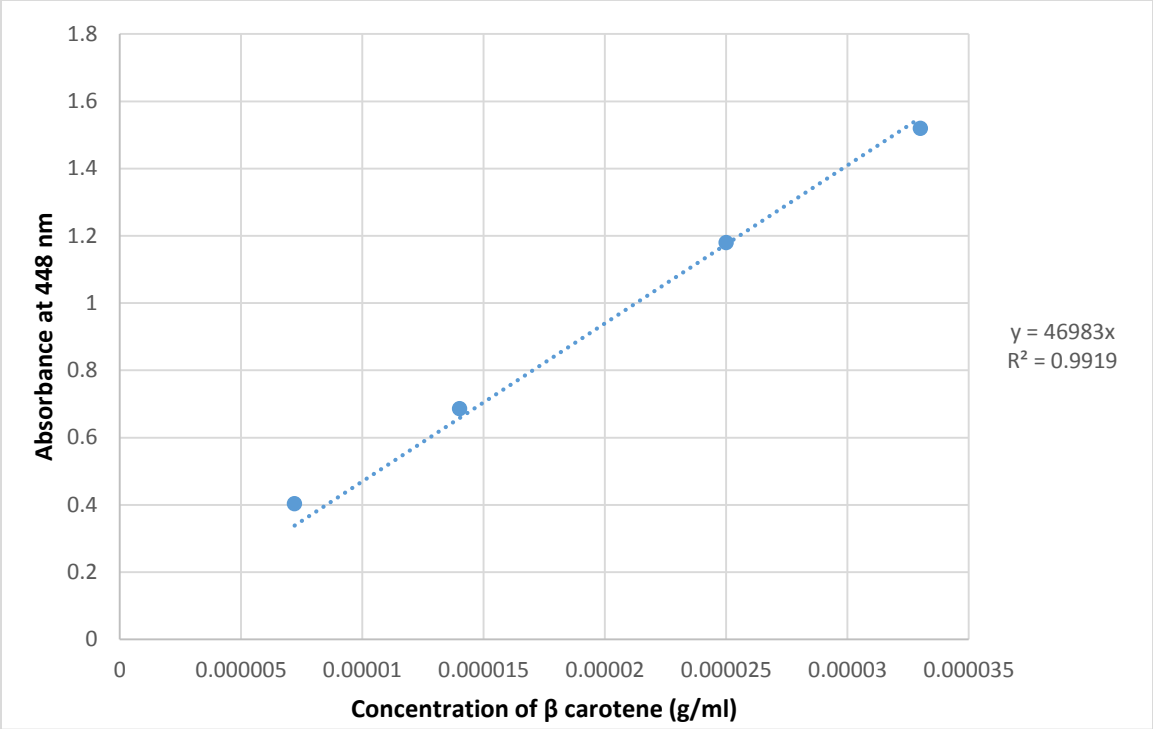
Calibration curve of β-carotene in Dichloromethane (DCM)



Calibration curve of β-carotene in simulated intestinal fluid.



Calibration curve of Zinc



Calibration curve of β-carotene in hexane